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AGENDA

SCIENTIFIC ADVISORY BOARD

March 13, 14, 15, 1974

1. Interim actions to be considered or confirmed:

- a. Administrative
- b. Executive Committee, SAB

2. Applications for Grants:

BOOK I - CANCER
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Dr. Henry Lynch
Creighton University
Under
Liebow

Retired
Andervant
Lynch.

Dr. David Stone
CTR Staff.

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THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

CONFIDENTIAL REPORT

SCIENTIFIC ADVISORY BOARD MEETING

NEW YORK, NEW YORK

OCTOBER 10-11-12, 1973

Dr. Sheldon C. Sommers, Chairman
Dr. Howard B. Andervont
Dr. Richard J. Bing
Dr. William U. Gardner
Dr. Robert J. Huebner
Dr. Leon O. Jacobson (10/10-11)
Dr. Hans Meier
Dr. John P. Wyatt

Scientific Director, CTR

Dr. Helmut R. R. Wakeham

Chairman, ITC

Mr. H. H. Ramm
Mr. W. T. Hoyt
Dr. Robert C. Hockett
Dr. John H. Kreisher
Dr. Frederic W. Nordsiek
Dr. Vincent F. Lisanti (10/10-11)
Mr. Leonard S. Zahn

President, CTR
Executive Vice President, CTR
Research Director, CTR
Associate Research Director, CTR
Associate Research Director, CTR
Research Associate, CTR
Consultant, CTR

The Chairman opened the meeting and reviewed changes in the composition of the staff and the Board since its last meeting. He noted that Dr. William U. Gardner's appointment as Scientific Director had become effective on July 1, 1973 and that on the same date Dr. Robert C. Hockett, who had been Acting Scientific Director, was appointed Research Director. Also that Drs. Cattell and Loosli had resigned from the Board and that Dr. Lynch had assumed the role of a Board member emeritus.

The Scientific Director advised that Dr. Averill A. Liebow, Professor and Chairman, Department of Pathology, at the University of California, San Diego, had agreed to join the Board, and that three other scientists were under consideration

1. The report of the meeting of the Scientific Advisory Board, held on March 14-15-16, 1973, was approved.
2. The report, actions, and recommendations of the June 13, 1973 meeting of the Executive Committee of the Scientific Advisory Board were approved and amended to authorize the staff to approve 776-AM, Elliot S. Vesell, M.D. up to an amount of \$34,730.00 less the \$8,500.00 previously approved by the Executive Committee (776-A) provided that Dr. Vesell provides a satisfactory report of progress and a modified application for continuation.

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3. The following administrative actions were approved:

- 381R2 Barbara B. Brown, Ph.D. A balance of \$2,248.75 remained after completion of this project and is therefore cancelled.
- 547-CR1 Joseph J. Guarneri, Ph.D. Permission was granted to carry forward the unexpended balance of \$1,578.43 to 547-CR2.
- 550-AMR2 John E. Craighead, M.D. An unexpended balance of \$584.62 was received and deposited.
- 603-AR2 Shirley L. Kauffman, M.D. Permission was granted to carry forward the unexpended balance of \$3,835.44 to 603-B.
- 603-B Permission was granted to extend this project from September 30 to December 31, 1973.
- 623-BR1 Walter B. Essman, Ph.D. A balance remained of \$450.00 after completion of this project and is therefore cancelled.
- 688-A Sheldon C. Sommers, M.D. An unexpended balance of \$11,202.98 was received and deposited.
- 702 Albert H. Niden, M.D. Through an administrative error the final quarter of this grant was not paid and, therefore, a check in the amount of \$4,253.75 was sent to Temple University to close out this project.
- 702-A A check in the amount of \$4,000.00 was received from Temple University, representing the estimated refund after Dr. Niden's transfer to Drew Postgraduate Medical School effective June 1, 1973. This money was deposited and a check drawn to Drew to aid Dr. Niden in starting up the project at that institution.
- 752R1S Erik Skinhoj, M.D. A supplement in the amount of \$256.00 was approved to pay for reprints from the July issue of the Journal of Applied Physiology.
- 763-A John W. Parker, M.D. Permission was granted to carry forward the unexpended balance of \$117.24 to 763-AR1.
- 763-AR1 Permission was granted to transfer \$800.00 from Supplies to Salaries.
- 763-A Permission was granted to extend this project from July 1 to December 31, 1973.
- 787-A Gary D. Friedman, M.D. An unexpended balance of \$5,182.65 remained from 787R1 and this was deducted from the present grant authorization of \$101,100.00, making the final authorization \$95,917.35.
- 808R1 Allen B. Cohen, M.D. An unexpended balance of \$2,305.94 was returned and deposited.
- 814R1 Una Smith, Ph.D. Permission was granted to transfer \$400.00 from Supplies to Travel.
- 864 Theodore Slotkin, Ph.D. Permission was granted to transfer \$3,025.00 from Supplies to Equipment.
- 877-S Herbert B. Herscovitz, Ph.D. Travel expenses for Dr. Herscovitz of \$119.23 were approved from Washington, D.C. to the CTR offices.

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Contract 3-B IIT Research Institute. After having been cancelled as of March 31, 1973 and funding requirements settled as of that date, a balance of \$12,398.32 remained which was cancelled. completion of this project and is therefore cancelled.

Contract 6-B After having been cancelled as of March 31, 1973 and funding requirements settled as of that date, a balance of \$16,168.44 remained which was cancelled.

The following authorizations were approved at a staff meeting on August 2, 1973:

709-CS Lucio Severi, M.D. This project was extended for the months of September and October 1973 in a pro rata amount of \$2,500.00 so that the grantee can continue his project until a decision as to his application is made at the SAB meeting in October.

836-AS Walter B. Essman, Ph.D., M.D. This project was extended for the month of October 1973 in a pro rata amount of \$3,334.00 so that the grantee can continue his project until a decision as to his application is made at the SAB meeting in October.

839RIS Edwin R. Fisher, M.D. This project was extended for the month of October 1973 in a pro rata amount of \$2,108.00 so that the grantee can continue his project until a decision as to his renewal application is made at the SAB meeting in October.

868S Herbert McKennis Jr., Ph.D. This project was extended for the month of October 1973 in a pro rata amount of \$5,000.00 so that the grantee can continue his project until a decision as to his renewal application is made at the SAB meeting in October.

875S Sheldon C. Sommers, M.D. This project was extended for the month of October 1973 in a pro rata amount of \$7,539.50 so that the grantee can continue his project until a decision as to his renewal application is made at the SAB meeting in October.

877S Herbert B. Herscowitz, Ph.D. This project was extended for the month of October 1973 in a pro rata amount of \$2,719.00 so that the grantee can continue his project until a decision as to his renewal application is made at the SAB meeting in October.

The following authorizations were approved:

Contract 2-E Microbiological Associates Incorporated. \$115.10 This amount was expended for testing five available inbred lines at Lakeview Hamster Colony for different aryl hydrocarbon hydroxylase levels. This contract was approved for \$157,000.00 at the Planning Committee meeting on December 8, 1972. Of this amount, a pro rata award of \$25,000.00 was authorized covering the period February 1 through March 31, 1973. The total contract as finally signed was for \$150,000.00 covering the period February 1, 1973 through January 31, 1974.

Contract 17 William E. Benedict, M.D. While traveling on contract business, Dr. Benedict incurred expenses totaling \$548.92 and was reimbursed.

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4. The following renewal applications were recommended for approval, subject to the determination by the Scientific Director as to priority and the amount to be funded:

709-CR1 Lucio Severi, M.D. \$19,948.00

814R2 Una Smith Ryan, Ph.D. \$35,373.00 Terminal.

836-AR1 Walter B. Essman, Ph.D., M.D. \$40,000.00

839R2 Edwin R. Fisher, M.D. \$24,978.00 Terminal.

843R2 A. Clifford Barger, M.D. \$50,000.00 Terminal.

868R1 Herbert McKennis Jr., Ph.D. \$60,000.00 Terminal.

869R1 Ronald P. Rubin, Ph.D. \$16,675.00

875R1 Harry L. Ioachim, M.D. (S. C. Sommers, M.D.) \$79,815.00 (See paragraphs 14 and 15, treating the request as two projects, one a supplement to 875 [Sommers] and one a new number 945 [Ioachim].)

877R1 Herbert B. Herscovitz, Ph.D. \$29,940.00

878R1 Paul Hamosh, M.D. \$28,265.00

889R1 Timothy J. Regan, M.D. \$45,500.00

891R1 Dr. Georg B. Neurath \$44,850.00

5. The following new applications were recommended for approval subject to the determination of the Scientific Director as to priority and the amount to be funded:

467-C Herbert B. Herscovitz, Ph.D. This project was extended for the month of October 1973 in a prorated amount of \$3,334.00 so that the application is made at the SAC meeting in October.

572-C Thomas C. Westfall, Ph.D. \$21,953.00

572-C Jay A. Levy, M.D. \$49,118.00

642-C Leonide Goldstein, D.Sc. \$33,350.00

702-C Albert H. Niden, M.D. \$25,618.00

741-B Joseph M. Lauweryns, M.D., Ph.D. \$24,556.00

787-B Gary D. Friedman, M.D. \$99,440.00

841-A Charles Mittman, M.D. \$36,608.00

864-A Theodore A. Slotkin, Ph.D. \$13,346.00

906 Robert E. Brooks, Ph.D. \$14,658.00

909 John A. Rosecrans, Ph.D. \$17,730.00

914 Michael E. Lamm, M.D. \$34,672.00

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- 915 Edward Bresnick, Ph.D. \$40,650.00
- 917 Patricia M. Hudgins, Ph.D. \$22,400.00
- 918 H. Fred Downey, Ph.D. \$15,290.00
- 923 Guenther Boden, M.D. \$36,206.00
- 928 Daniel B. Rifkin, Ph.D. \$36,077.00
- 929 Edward Leete, Ph.D. \$23,709.00
- 933 Charles R. Shaw, M.D. \$35,000.00
- 935 William H. Fishman, Ph.D. \$62,853.00
- 936 Branslav Vidic, Sc.D. \$40,738.00
- 937 Carroll E. Cross, M.D. \$44,650.00
6. The following new application was deferred pending a site visit and receipt of additional information:
- 912 Sam G. McClugage, Ph.D. \$38,094.00
7. The following new applications were rated for disapproval:
- 603-C Shirley L. Kauffman, M.D. \$30,521.00
- 815-B John R. Esterly, M.D. \$34,482.00
- 833-A A. Stanley Weltman, Ph.D. \$58,386.00
- 888 T. J. Yang, Ph.D. \$43,692.00
- 904 David Aperia, Ph.D. \$50,496.00
- 905 Edward P. Domino, M.D. \$52,885.00
- 907 Ines Mandl, Ph.D. \$38,717.00
- 908 Sanford E. Leeds, M.D. \$56,485.00
- 910 James M. Ramsey, M.D. \$10,859.00
- 911 Wistar Institute \$83,075.00
- 913 Anthony J. Sbarra, Ph.D. \$65,100.00
- 916 Robert A. Goldstein, M.D. \$29,900.00
- 919 John R. Daniels, M.D. \$51,216.00
- 920 William F. Benedict, M.D. \$25,700.00
- 921 Theodore N. Finley, M.D. \$90,712.00
- 922 Joseph C. Arcos, M.D. \$49,738.00

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924	John C. Houck, Ph.D.	\$36,098.00
925	L. G. S. Rao, Ph.D.	\$3,950.00
926	Budh Dev Bhagat, Ph.D.	\$31,360.00
927	David J. Wilson, Ph.D.	\$8,973.00
930	Andrew Sivak, Ph.D.	\$54,838.00
931	Gerald M. Rosen, Ph.D.	\$12,156.00
932	W. LeRoy Heinrichs, M.D., Ph.D.	\$73,108.00
934	Wendell M. Stanley, Ph.D.	\$65,420.00

8. The following proposal for contract renewal was recommended for approval subject to the determination of the Scientific Director as to priority and the amount to be funded:

Contract 2-F Microbiological Associates Incorporated \$137,500.00 for a period of eleven months.

9. The following proposal for contract continuation was rated for disapproval:

Contract 5-D Bio-Research Consultants, Inc. \$499,000.00

10. The following proposal for contract continuation was deferred and the Executive Committee of the Advisory Board was authorized to act as necessary after a staff commentary and recommendation are distributed to the entire Board and each member has had an opportunity to express an opinion:

Contract 4-D Bio-Research Institute Incorporated \$157,000.00

11. The Board recommended that the staff undertake to contract with Richard A. Lerner, M.D. to expand the work he is now conducting (Contract 16) at a cost not to exceed \$60,000.00 for one year beginning January 1, 1974.

12. The status of twin studies was reviewed by Dr. Gardner who described his recent meeting with Dr. Rune Cederlöf and Dr. Lars Friberg in Stockholm, Sweden. The Board recommended approval of up to a total of \$50,000.00 for the staff to apply to:

1. Preliminary costs to cover arrangements for further exploratory work by Dr. Cederlöf and his associates at The Karolinska Institute, and
2. Underwriting an organizational meeting of approximately 25 people, representing twin registries and data from various places to be held during the week of December 10, 1973 in Miami Beach.

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13. The next meeting of the Scientific Advisory Board was scheduled for March 13-14-15, 1974 in Scottsdale, Arizona. The staff was requested to invite Dr. Carrie Whitmire and Dr. Frank Dixon to attend.

14. Subsequent to the meeting, of the renewal applications recommended for approval and referred to in paragraph 4, the Scientific Director determined that the following should be funded in the respective amounts indicated:

709-CS2	Lucio Severi, M.D.	\$7,500.00 less \$2,500.00 (709-CS).	(See page 3.) Terminal.
814R2	Una Smith Ryan, Ph.D.	\$35,373.00	Terminal.
836-AR1	Walter B. Essman, Ph.D., M.D.	\$44,000.00 less \$3,334.00 (836-AS).	(See page 3.)
839R1S2	Edwin R. Fisher, M.D.	\$12,489.00 less \$2,108.00 (839R1S).	(See page 3.) Terminal.
843R2	A. Clifford Barger, M.D.	\$50,000.00	Terminal.
868S2	Herbert McKennis Jr., Ph.D.	\$30,000.00 less \$5,000.00 (868S).	(See page 3.) Terminal.
869R1	Ronald P. Rubin, Ph.D.	\$16,675.00	Terminal.
875S2	Sheldon C. Sommers, M.D.	\$15,079.00 supplement for the months of November and December 1973.	(See 875R1, application by Ioachim and Sommers referred to in paragraph 4.)
877R1	Herbert B. Herscowitz, Ph.D.	\$29,940.00 less \$2,719.00 (877S).	(See page 3.)
878R1	Paul Hamosh, M.D.	\$28,265.00	
889R1	Timothy J. Regan, M.D.	\$45,500.00	
891R1	Dr. Georg B. Neurath	\$44,850.00	

15. Subsequent to the meeting, of the new applications recommended for approval and referred to in paragraph 5, the Scientific Director determined that the following should be funded in the respective amounts indicated:

467-C	Thomas C. Westfall, Ph.D.	\$21,953.00	
572-C	Jay A. Levy, M.D.	\$40,000.00	
642-C	Leonid Goldstein, D.Sc.	\$33,350.00	Terminal.
702-C	Albert H. Niden, M.D.	\$25,618.00	No assurance of further support.
741-B	Joseph M. Lauweryns, M.D., Ph.D.	\$24,556.00	No assurance of further support.

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787-B Gary D. Friedman, M.D. \$99,440.00
841-A Charles Mittman, M.D. \$32,238.00 Terminal.
864-A Theodore A. Slotkin, Ph.D. \$13,346.00 No assurance of further support.
906 Robert E. Brooks, Ph.D. \$14,658.00 No assurance of further support.
909 John A. Rosecrans, Ph.D. \$17,730.00
914 Michael E. Lamm, M.D. \$34,672.00
918 H. Fred Downey, Ph.D. \$15,290.00
923 Guenther Boden, M.D. \$36,206.00
928 Daniel B. Rifkin, Ph.D. \$36,077.00
929 Edward Leete, Ph.D. \$23,709.00 No assurance of further support.
933 Charles R. Shaw, M.D. \$35,000.00
936 Branislav Vidic, S.D. \$36,256.00
937 Carroll E. Cross, M.D. \$44,650.00
945 Harry L. Ioachim, M.D. \$40,000.00 No assurance of further support.
(See 875R1, application by Ioachim and Sommers, referred to in paragraph 4.)

16. Subsequent to the meeting, of the new applications recommended for approval and referred to in paragraph 5, the Scientific Director determined that the following should be deferred pending further information:

935 William H. Fishman, Ph.D. \$36,256.00

17. Subsequent to the meeting, of the contract proposals recommended for approval and referred to in paragraph 8, the Scientific Director determined that the following should be funded in the respective amount indicated:

Contract 2-F Microbiological Associates Incorporated \$137,500.00 for a period of eleven months.

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ADMINISTRATIVE REPORT

Contract 17-A By mail action of the Executive Committee, a service contract to William F. Benedict, M.D. was approved. \$15,000.00

Contract 18S Reference cigarettes. The Scientific Director approved the contract in a total amount of \$139,500.00, requiring a supplementary appropriation of \$ 4,500.00

519-B Louis A. Soloff, M.D. was authorized to carry forward the unused balance on hand at May 31, 1973 to 519-BR1.

572-C Jay A. Levy, M.D. Permission was granted to activate this grant on September 1, 1973, to terminate on December 31, 1974.

646-AR1 Robert C. Rosan, M.D. An unexpended balance of \$652.50 was returned and deposited.

702-A Alfred H. Niden, M.D. An unexpended balance of \$2,564.32 was returned by Temple University and deposited.

763-AR1 John W. Parker, M.D. Authorization was given to re-budget \$650.00 from Materials & Supplies to Travel.

776M2R1S1 Elliot S. Vesell, M.D. An extension was authorized for the period December 31, 1973 to June 30, 1974, and permission granted to carry forward a balance of \$5,478.49.

787-A Gary D. Friedman, M.D. was authorized to carry forward approximately \$4,000-5,000 as of January 31, 1974 to Grant 787-B.

814R1 Una Smith Ryan, Ph.D. was authorized to carry forward the balance on hand (approximately \$2,000) as of December 31, 1973 to 814R2 for the purchase of a dishwashing machine.

833R1 A. Stanley Weltman, Ph.D. An extension was authorized for the period December 31, 1973 to June 30, 1974, and permission was granted to carry forward a balance of \$5,478.49.

868 Herbert McKennis Jr., Ph.D. was authorized to carry forward an unused balance on hand at April 1, 1974.

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869

Ronald P. Rubin, Ph.D. An extension was authorized for the period December 31, 1973 to June 30, 1974 with no additional funds.

869R1

Approval was given to transfer this renewal award from the State University of New York to Virginia Commonwealth University, to be activated July 1, 1974.

Contract 4S

Meloy Laboratories, Inc. Authorization was given to deliver the unused balance (\$3,333.36) of reference cigarette condensate to Hans Meier, D.V.M. (Grant 758-BR1) instead of Bio-Research Institute, Inc. (Contract 4-B).

Contract 13S

Meloy Laboratories, Inc. An unspent balance of \$370.00 is cancelled due to Contract 13 (Microbiological Associates Inc.) being terminated.

Contract 19

Process & Instruments Corporation. \$62,500.00 Termed Contract 10 in the March 14-16, 1973 minutes, it should have been Contract 19.

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THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

MEETING

EXECUTIVE COMMITTEE OF THE SCIENTIFIC ADVISORY BOARD

DECEMBER 4, 1973

NEW YORK, N. Y.

Attendance

Sheldon C. Sommers, Chairman
Richard J. Bing
Leon O. Jacobson
John P. Wyatt

H. H. Ramm
W. T. Hoyt
William U. Gardner
Robert C. Hockett
Frederic W. Nordsiek

President, CTR
Executive Vice President, CTR
Scientific Director, CTR
Research Director, CTR
Associate Research Director

The following recommendations, items 1 through 4, were made subject to the determination by the Scientific Director as to priority and the amount to be funded.

1. Contract 10. \$45,000.00 as a supplement for staff to allocate for developmental work and for rebuilding smoking machines at Process and Instruments Corporation.
2. Contract 20. \$40,000.00 for use in the development of a prototype of a smoking machine that will produce a continuous stream of smoke for biological purposes.
3. Contract 18. In view of estimates from the University of Kentucky indicating sharply increased costs for production of reference cigarettes, an increase of \$100,000.00 (see March 14-16 SAB report of actions) to a total amount not to exceed \$200,000.00.
4. Bio-Research Consultants, Inc. proposal 5-D, which was disapproved at the October 1973 Board meeting, was presented for reconsideration at a reduced cost and was again disapproved.

Bio-Research Institute, Incorporated contract proposal 4-D, which was deferred at the October 1973 Board meeting, was discussed in the light of a report by The Council's staff. The staff was authorized to negotiate a new contract not to exceed \$50,000.00 with Bio-Research Institute, Incorporated.

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5. It was decided that the staff should dispense \$3,336.36 remaining unspent in the Meloy Laboratories, Inc. condensate supplement to Contract 4-C, to other grantees or contractors having need for it.

6. The staff reported that the Elliot S. Vesell grant award (776-AM) was finally fixed at a total figure of \$33,500 rather than \$34,730.00 (see October 10-12, 1973 report of Board actions).

7. Dr. Gardner reported that Henry T. Lynch, M.D., Professor and Chairman, Department of Preventive Medicine and Public Health at Creighton University, had accepted membership on the Scientific Advisory Board.

8. It was decided that per diems for the Scientific Advisory Board should henceforth be \$250.00 instead of \$150.00.

9. Eight inquiries suggesting outlines of possible project proposals were considered. Of this number, three were rated sufficiently interesting and appropriate to warrant encouraging formal applications. One was deferred in order to obtain more specific information.

10. Subsequent to the meeting, of the authorizations referred to in (1), (2), (3), and (4), the Scientific Director determined that the following should be approved in the respective amounts indicated:

1. Contract 10 - Up to \$45,000 as a supplement for staff to allocate for developmental work and for rebuilding smoking machines at Process and Instruments Corporation.

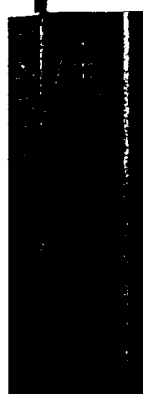
2. Contract 18 - Up to \$35,000 increase in the original \$100,000 appropriated for the production of reference cigarettes (see March 14-16 SAB report of actions).

3. Bio-Research Institute, Incorporated. Contract proposal 4-D up to \$50,000 for a new contract with a modified project.

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APPLICATIONS TO BE CONSIDERED

March 13, 14, 15, 1974

Grant No.	Principal Investigator	Amount Requested	Current Grant	Committee
<u>CANCER</u>				
922M	Arcos	\$55,189		Huebner, Meier, Sommers
972	Friedman	\$39,201		Gardner, Huebner, Meier
944	Glick	\$60,573		Jacobson, Huebner, Meier
946	Herrmann	\$11,335		Gardner, Huebner, Meier
956	Kleinerman	\$36,904		Gardner, Huebner, Meier
980	Lai	\$29,923		Gardner, Huebner, Meier
766AR1	Lerner	\$65,378	\$52,385	Gardner, Huebner, Meier
951	Meier	\$25,461		Gardner, Huebner, Jacobson
973	Rasmussen	\$34,645		Gardner, Huebner, Meier
950	Rosenkranz	\$14,134		Huebner, Meier, Sommers
967	Roszman	\$29,824		Gardner, Huebner, Meier
966	Sloane	\$35,090		Gardner, Huebner, Jacobson
979	Triolo	\$48,493		Gardner, Huebner, Meier

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Applications

2

Grant No.	Principal Investigator	Amount Requested	Current Grant	Committee
<u>EPIDEMIOLOGY</u>				
826A	Dawber	\$54,625		Gardner, Sommers, Lynch
968	Friberg	\$15,000		Gardner, Sommers, Lynch
971	Honeyman	\$54,940		Bing, Gardner, Lynch
965	Kjellstrom	\$12,756		Gardner, Jacobson, Lynch
941	Lynch	\$30,360 Part I \$29,176 Part II		Gardner, Huebner, Jacobson
978	Mimura	\$43,119		Gardner, Sommers, Lynch
953	Rantasalo	\$61,235		Gardner, Jacobson, Lynch
<u>PULMONARY</u>				
959	Altwickier	\$78,640		Gardner, Liebow, Wyatt
976	Ayres	\$30,061		Gardner, Liebow, Wyatt
764C	Cochrane	\$16,560	\$11,000 Terminal	Gardner, Liebow, Wyatt
954	Cohen	\$46,045		Gardner, Liebow, Sommers
962	Cugell	\$26,857		Liebow, Sommers, Wyatt
547D	Guarneri	\$51,835	\$22,944	Jacobson, Liebow, Wyatt
908M	Leeds	\$44,134		Gardner, Liebow, Wyatt
948	Lentz	\$20,626		Gardner, Liebow, Sommers
961	Michaelli	\$96,603		Liebow, Meier, Wyatt

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Applications

3

Grant No.	Principal Investigator	Amount Requested	Current Grant	Committee
<u>PULMONARY</u> (cont'd)				
938	Sackner	\$91,465		Liebow, Sommers, Wyatt
975	Wallace	\$60,201		Liebow, Sommers, Wyatt
958	Webb	\$27,372		Gardner, Liebow, Wyatt
901M	Weinbaum	\$38,163		Liebow, Meier, Wyatt
<u>CARDIOVASCULAR</u>				
310R13	Bing	\$45,293	\$39,893	Jacobson, Liebow, Sommers
939	Mason	\$41,995		Bing, Jacobson, Wyatt
952	McDonald	\$43,911		Bing, Jacobson, Sommers
519C	Soloff	\$75,873	\$62,500	Bing, Jacobson, Liebow
974	Vatner	\$31,595		Bing, Jacobson, Wyatt
<u>PHARMACOLOGY</u>				
623C	Essman	\$22,567	\$21,579	Bing, Gardner, Sommers
970	McKennis	\$38,662		Bing, Jacobson, Sommers
949	Sastry	\$12,806		Bing, Gardner, Sommers
969	Timasheff	\$52,450		Gardner, Jacobson, Sommers
943	Volle	\$22,028		Bing, Jacobson, Liebow
960	Giri	\$23,865		Bing, Huebner, Meier
912	McClugage, Jr.	\$38,094		Bing, Jacobson, Meier

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CANCER

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#922M - ARCO8

1003545106

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

February 7, 1974

Grant application No. 922M

CANCER

To: The committee comprising Drs. Huebner, Meier, Sommers

Subject: Joseph C. Arcos, D.Sc., Tulane University, New Orleans
Modified application No. 922M
"Synergistic Effects of Polycyclic Hydrocarbons and
Nitrosamines in Pulmonary Carcinogenesis. Potential
Repressors of Metabolic Activation of Nitrosamines"

History

This proposal is a modification of application No. 922, considered by the SAB at its October 1973 meeting. The action then was to defer for a site visit and modification of the proposal, both now completed. The SAB committee for application No. 922 was Drs. Andervont, Huebner and Meier.

Request

Application No. 922M requests \$55,189, plus two additional years in larger amounts.

Document Submitted

You have received the application dated 1/17/74 from Dr. Kreisher with his memorandum dated February 5, 1974.

Comment

To refresh recollection, enclosed are copies of evaluations by Dr. W. F. Dunning and Dr. Richard E. Kouri of the earlier application No. 922.

Also enclosed are copies of comments by SAB members Bing, Huebner and Meier on the earlier application.

F.W.N.
F.W.N.

FWN:wg
Encls.

1003545107

W. F. Dunning, Ph.D.
Papanicolaou Cancer Res. Inst
October 3, 1973

Grant Application # 922 - Arcos

Title: Synergistic effects of polycyclic hydrocarbons and nitrosamines in pulmonary carcinogenesis. Potential repressors of metabolic activation of nitrosamines.

Comments:

1. Investigator appears to be well qualified to conduct the proposed research program.
2. The experimental plan is adequate to determine the extent of synergism between the hydrocarbons 3,4-Benzopyrene and 3,4-Benzofluoranthene and nitrosamines Dimethylnitrosamine, Methyl-ethylnitrosamine and N-Nitrosopiperidine.
3. The proposal to test the anti-carcinogenic effect of B-Naphthoflavone and Pregnenolone-16 α -carbonitrile is well supported by the working hypothesis.
4. The estimated time of 4 years for completion seems practical.
5. The budget is very reasonable for the maintenance of 700 rats with complicated long term experimental protocols and the eventual preparation and analysis of necropsy and histopathological data.

CTR - bcc: Drs. Andervont, Huebner, Meier
Drs. Gardner, Hockett, Mr. Hoyt

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9227

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8865

JAN 23 1974

Application for Research Grant (Revised) _____ Date: 3/17/74
(Use extra pages as needed)

1. Principal Investigator (give title and degrees):

Joseph C. Arcos and Mary F. Argus
Professor of Medicine Professor of Medicine
L. Chem., Ch. E., D. Sc. B. S., M. S., Ph. D.

Mailing address:

Seamen's Memorial
Research Laboratory
USPHS Hospital
210 State Street
New Orleans, La. 70118

2. Institution & address:

Tulane University School of Medicine
1430 Tulane Ave.
New Orleans, La. 70112

3. Department(s) where research will be done or collaboration provided:

Department of Medicine

4. Short title of study: Synergistic effects of polycyclic hydrocarbons and nitrosamines in pulmonary carcinogenesis. Potential repressors of metabolic activation of nitrosamines.

5. Proposed starting date: 3/1/74

6. Estimated time to complete: 3 years

7. Brief description of specific research aims:

First, to explore experimentally the very definite possibility (see 8. Background and brief statement of working hypothesis) that a significant proportion of the lung tumor incidence of cigarette smokers is due to synergism between hydrocarbons and nitrosamines present in the smoke, and to attempt to elucidate the mechanism of this synergism by determining how the enzyme systems metabolizing these two types of compounds are related to each other. A further ramification of such studies is that the oral nitrosamine intake of smokers (as dietary nitrosamine contaminants, or pro-nitrosamine additives, such as nitrite together with ubiquitously present secondary amines in meat products) could play an important role in their high lung cancer incidence.

Second, to investigate whether certain nontoxic inhibitors of nitrosamine metabolism — a process known to be required for carcinogenesis by these agents — also inhibit nitrosamine carcinogenesis and decrease lung tumor incidence by disrupting the hydrocarbon-nitrosamine synergism.

In the present study we propose to investigate the synergism toward lung tissue of the highly carcinogenic hydrocarbon, 3-methylcholanthrene (MC), and the carcinogenic nitrosamine, dimethylnitrosamine (DMN). We will determine the combined carcinogenic effect of the two compounds in an inbred strain of mice in which aryl hydrocarbon hydroxylase (AHH), the enzyme system which both activates and detoxifies MC, is present or inducible, and in a

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strain of mice in which this enzyme system is noninducible. Throughout the time course of this study, the effect of administration of the compounds on the tissue level of AHH and the level of DMN-demethylase, a mixed function oxidase system capable of converting DMN to a proximate carcinogen, will be determined. If the synergism is established to be strain dependent, the inheritability of the susceptibility will be determined in first generation hybrid crosses of the two strains. Also within the framework of the present study we intend to determine the inhibitory effect of β -naphthoflavone and pregnenolone-16 α -carbonitrile on the carcinogenic activity of DMN; these two agents are powerful inhibitors of DMN metabolism, and are virtually nontoxic:

8. Background and brief statement of working hypothesis:

A.) Hydrocarbon-nitrosamine synergism. — A previous study (1) in our laboratory has shown that administration of 3-methylcholanthrene (MC) inhibits liver tumor induction in rats by simultaneously administered dimethylnitrosamine (DMN). In a series of investigations (2-6) we have established that this is due to inhibition in the liver of biosynthesis of the enzyme, DMN-demethylase, the key enzyme transforming this nitrosamine to its actual carcinogenic metabolite. In fact DMN-demethylase has proved to be one of the rare mixed function oxidases, the de novo synthesis of which is repressed by MC, phenobarbital and numerous other so-called "enzyme inducers". By studies with 58 polynuclear hydrocarbons we have established that the repression of DMN-demethylase synthesis, just as the previously-studied induction of other microsomal enzyme systems (7), is a function of molecular size and planarity; and what is most striking, these two effects show a mirror image relationship (5, 6). This finding, together with the fact that a much shorter latency is required for enzyme induction than for enzyme repression (e.g., 5-6 hours vs. 12-13 hours with MC), provide support for our previously suggested theoretical model that inducible and repressible microsomal oxidase levels are regulated by cascade-coupled operons (8).

An unexpected and important finding of the carcinogenesis study (1) was that while the hepatocarcinogenicity of DMN is inhibited by the simultaneous oral administration of MC, the two agents have a powerful synergistic effect in lung tumor induction. Indeed, whereas at the levels used in the study neither the nitrosamine nor the hydrocarbon alone was carcinogenic in the lung, combined administration of the two agents brought about a 15% pulmonary tumor incidence. More recently Cardesa et al. (9) have demonstrated a syncarcinogenic effect with MC and DMN in the lungs of Swiss mice. The practical significance of these results lies in the facts (a) that MC is known to be very similar or identical in action to the two potent hydrocarbon carcinogens, 3,4-benzopyrene and 3,4-benzofluoranthene, present in cigarette smoke (10), and (b) that DMN, methylethyl-nitrosamine, and N-nitrosopiperidine are also present in the smoke (11) at a total level of similar order of magnitude as the hydrocarbons (12).

The above considerations point to the high probability that a significant proportion of the lung tumor incidence of smokers is due to synergism between carcinogenic hydrocarbons and nitrosamines present in the smoke, rather than to hydrocarbons alone. When DMN is administered to rats at a higher total dose than in the above-cited study (1), lung tumors are produced

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by DMN alone (13). Thus since the lung is a tissue target of DMN, it is not unreasonable to suppose that administration of DMN brings about deletion of various microsomal mixed function oxidases in that tissue just as is known to happen in the liver (14). This would result in a decrease of the rate of MC hydroxylation via AHH in the lung, whereby higher topical level of hydrocarbon would become available for tumorigenesis. Another possible explanation is that DMN induces AHH synthesis and thus the rate of MC activation is accelerated. These possibilities will be explored by measuring the rate of in vitro MC metabolism in lung tissue during the time-course of prolonged DMN and MC administration.

On the other hand the possibility can not be ignored that repressibility of DMN-demethylase in the lung may be greatly decreased in the adult animals in which the carcinogenicity studies have been carried out, as opposed to the response in weanling animals which have been used in the enzyme studies. It is well known that the inducibility of the synthesis of various hepatic mixed function oxidases by polycyclic hydrocarbons gradually decreases as the animals progress from the weanling to the adult stage. The requirement regarding age for inducibility by hydrocarbons in lung tissue does not appear to have been studied. In the absence of proof to the contrary it cannot be entirely excluded at present that in lung tissue the repressibility of DMN-demethylase may entirely disappear with animal age. The possibility exists that DMN-demethylase synthesis in the lung may even be induced by prolonged ingestion of MC in adult animals. Thus the level of DMN-demethylase activity will also be determined in lung tissue during the time course of prolonged DMN and MC administration.

The lung tissue for these enzyme (AHH and DMN-demethylase) activity determinations will be provided by animals treated with MC and DMN in groups paralleling a study of the syncarcinogenic effects of these two compounds toward the lung. Animal test systems having very special characteristics to aid in elucidating relationships between susceptibility to chemical carcinogenesis and ability to metabolize enzymatically chemical carcinogens have recently been defined. It has been established that induction or increase in AHH activity occurs only in certain inbred strains of mice (15, 16) and these strains are more susceptible to MC-induced subcutaneous tumor than are strains in which AHH is noninducible (17, 18). For example, the C57BL/6 (B6) mouse strain in which the hepatic AHH is inducible in all mice gave a 83% tumor incidence for MC-induced subcutaneous tumors, while the DBA/2 (D2) strain in which hepatic AHH is noninducible in all mice gave only a 7% tumor incidence (18). It was further shown that the enzyme induction segregated as a single autosomal dominant gene in crosses of strains C57BL/6 and DBA/2 (15, 16), and that the genetically controlled presence of the inducibility of AHH activity in backcrosses between C57BL/6 and DBA/2 strains correlated with susceptibility to MC-induced subcutaneous tumors.

Because of these unique characteristics of these two strains of mice (C57BL/6 and DBA/2) we plan to employ them in the presently proposed studies on the possible synergism between hydrocarbon and nitrosamine carcinogens (specifically MC and DMN) on lung tumorigenesis. These strains will

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especially lend themselves to an attempt to elucidate (a) the mechanism of such a synergism, (b) how it is related to induction (or repression) of the enzyme systems involved with metabolism of the carcinogenic agents, (c) how these enzyme systems themselves are interrelated, (d) to provide evidence for or against our hypothesis that inducible and repressible microsomal oxidase levels are gene regulated by cascade-coupled operons, and (e) to indicate inheritability of susceptibility to the action of certain agents and combinations of agents.

B.) Anti-carcinogenic effects of β -naphthoflavone and pregnenolone-16 α -carbonitrile. — We have demonstrated that not only polycyclic hydrocarbons but also phenobarbital (2, 3), as well as aminoacetonitrile, β -naphthoflavone and pregnenolone-16 α -carbonitrile (6) inhibit the critical metabolic route of dimethylnitrosamine leading to its actual carcinogenic metabolite. Kunz *et al.* (19) reported that administration of phenobarbital inhibits the carcinogenicity of diethylnitrosamine, and Hadjiolov (20) found that administration of aminoacetonitrile inhibits the carcinogenicity of dimethylnitrosamine.

It is well established that β -naphthoflavone inhibits the carcinogenic action of certain hydrocarbons (e.g., 21, 22). It is unknown as yet whether β -naphthoflavone and pregnenolone-16 α -carbonitrile inhibit carcinogenesis by nitrosamines, but from the established evidence that they powerfully inhibit the obligatory metabolic route of dimethylnitrosamine, it is expected that they will be found to be potent inhibitors of nitrosamine carcinogenesis. Also, since it has been shown that agents repressing DMN-demethylase synthesis are inducers of other microsomal mixed function oxidases (5, 6), β -naphthoflavone and pregnenolone-16 α -carbonitrile can be expected to be inducers of AHH. Moreover neither of these two compounds are carcinogenic, toxic, or appear to have any hormonal or physiological activity. Thus, they show definite promise as effective agents to disrupt the hydrocarbon-nitrosamine pulmonary synergism.

9. Details of experimental design and procedures:

Hydrocarbon-nitrosamine synergism — The syncarcinogenic effects of 3-methylcholanthrene (MC) and dimethylnitrosamine (DMN) will be carried out in male C57BL/6 and DBA/2 mice, 8 weeks old at the beginning of the experiment. Dosage levels and routes of administration will follow those used in mice by Cardesa *et al.* (9), with the exception that olive oil will be replaced with 1% aqueous methylcellulose as the vehicle for MC (1). Thus mice will receive 6 mg DMN/kg body weight once weekly, injected intraperitoneally as a 0.024% DMN solution in 0.9% saline; MC (as a 0.3% suspension in 1% methylcellulose) will be administered intragastrically at a level of 1.5 mg MC/kg body weight, 3 times weekly; administration of the carcinogens will be for 10 weeks. For each strain (C57BL/6 and DBA/2) of mice the following groups of 60 animals each will be employed: (a) controls receiving the administration vehicles only, (b) mice receiving MC only, (c) mice receiving DMN only, and (d) mice receiving both MC and DMN.

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After the schedules of carcinogen administration will be terminated, the animal groups will be maintained for an additional 10 months (9). Following the routine procedure in our laboratory, during this observational period the animals will be checked twice daily and the state of their health carefully followed. During this observational period any animal showing clear evidence of the presence of a tumor (*i. e.*, large subcutaneous or abdominally localized), or morbidity, will be sacrificed, submitted to complete autopsy, and the tissue samples fixed in neutral formaline for histopathology. All survivors will be sacrificed at the end of the observational period and treated as above. Histopathology will be carried by Dr. Joseph Simon, Professor of Pathology, College of Veterinary Medicine, University of Illinois, Urbana, and by Dr. Cornelia Hoch-Ligeti, Chief, Pathology Service and Research Laboratory, Veterans Administration Center, Martinsburg, W. Va., with whom we have had excellent collaboration for the last 15 years.

Assay of AHH and DMN-demethylase enzyme levels — The level of activity of both enzymes, AHH and DMN-demethylase, will be determined, at 1, 3, 5, 8 and 10 weeks, in liver and lung of mice submitted to the following regimes: (a) C57BL/6 mice receiving MC, (b) C57BL/6 mice receiving DMN, (c) C57BL/6 mice receiving MC + DMN, (d) control C57BL/6 mice receiving the administration vehicles only, (e) DBA/2 mice receiving MC, (f) DBA/2 mice receiving DMN, (g) DBA/2 mice receiving MC + DMN, and (h) control DBA/2 mice receiving the administration vehicles only. Dose levels and routes of administration of the carcinogens will parallel those described for the synergism experiments above.

Appropriate amounts of liver or lung tissue will be pooled for the isolation of microsomes, which will be carried out by the usual method in 0.25 M sucrose (2). In the assay of DMN-demethylase, the demethylation reaction and measurement of HCHO will follow the procedures routinely used in our laboratory (2).

Because in these studies MC will serve a possible dual role, *i. e.*, not only as an "enzyme inducer-repressor" but also as an actual carcinogenic agent, we believe that MC should be employed as substrate in the *in vitro* determination of AHH activity levels. Thus the total polar MC metabolites formed by isolated microsomal preparations in the standard incubation medium will be measured fluorimetrically by adapting to this hydrocarbon the method of Wattenberg *et al.* (23) as modified by Conney and Levin (24).

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Anti-carcinogenic effects of β -naphthoflavone and pregnenolone-16 α -carbonitrile. — As it has already been described in "Background and brief statement of working hypothesis" these two compounds are powerful inhibitors of the demethylation of DMN, the metabolic route which is the controlling step of tumor induction by this agent. Thus, combined administration of either of these two compounds with DMN and/or MC should decrease their carcinogenicity.

Since both β -naphthoflavone and pregnenolone-16 α -carbonitrile are costly compounds, it will be necessary to determine first the lowest dietary levels in the mouse at which saturation of DMN-demethylase inhibition is reached. In a preliminary study in the rat of the dose-dependence of inhibition of DMN-demethylase biosynthesis by *in vivo* administered pregnenolone-16 α -carbonitrile

we found that 22-25 mg/day/kg body weight is slightly beyond the saturation dose for inhibition. This is also the appropriate dose for β -naphthoflavone to reach saturation level, as indicated by a comparison of our preliminary data with the results of Cantrell and Bresnick (25).

Once the dose level appropriate for the mouse has been confirmed, this level of β -naphthoflavone or pregnenolone-16 α -carbonitrile will be incorporated into the diet of mice receiving MC, DMN, or MC + DMN, in the regimes described for the synergism studies. Administration of an inhibitor will start 1 week prior to the beginning of carcinogen administration. As in the synergism experiments, following termination of the administration schedule the animals will be maintained for an additional observational period of 10 months. Maintenance of the animals, autopsies, and histopathology will be as above.

References:

- (1) Hoch-Ligeti, C., Argus, M. F., and Arcos, J. C.: "Combined Carcinogenic Effects of Dimethylnitrosamine and 3-Methylcholanthrene in the Rat". *J. Natl. Cancer Inst.* **40**, 535 (1968).
- (2) Venkatesan, N., Arcos, J. C., and Argus, M. F.: "Differential Effect of Polycyclic Hydrocarbons on the Demethylation of the Carcinogen Dimethylnitrosamine by Rat Tissues". *Life Sci.* **7** (Part I), 1111 (1968).
- (3) Venkatesan, N., Argus, M. F., and Arcos, J. C.: "Mechanism of 3-Methylcholanthrene-induced Inhibition of Dimethylnitrosamine Demethylase in Rat Liver". *Cancer Res.* **30**, 2556 (1970).
- (4) Venkatesan, N., Arcos, J. C., and Argus, M. F.: "Amino Acid Induction and Carbohydrate Repression of Dimethylnitrosamine Demethylase in Rat Liver". *Cancer Res.* **30**, 2563 (1970).
- (5) Argus, M. F., Valle, R. T., Venkatesan, N., Buu-Hoi, N. P., and Arcos, J. C.: "Molecular-size-dependent effects of polynuclear hydrocarbons on mixed-function oxidases: possible action on cascade-coupled operons". *Proc. 1st Europ. Biophys. Congr.* **E1/38**, 187 (1971).
- (6) Arcos, J. C., Argus, M. F., and Buu-Hoi, N. P.: "Repression of dimethylnitrosamine demethylase by polynuclear hydrocarbons and other compounds". *Federation Proc.* **32**, 702 (1973).
- (7) Arcos, J. C., Conney, A. H., and Buu-Hoi, N. P.: "Induction of Microsomal Enzyme Synthesis by Polycyclic Aromatic Hydrocarbons of Different Molecular Sizes". *J. Biol. Chem.* **236**, 1291 (1961).
- (8) Venkatesan, N., Arcos, J. C., and Argus, M. F.: "Induction and Repression of Microsomal Drug-metabolizing Enzymes by Polycyclic Hydrocarbons and Phenobarbital: Theoretical Models". *J. theor. Biol.* **33**, 517 (1971).
- (9) Cardesa, A., Pour, P., Rustia, M., Althoff, J., and Mohr, U.: "The Syncarcinogenic Effect of Methylcholanthrene and Dimethylnitrosamine in Swiss Mice". *Z. Krebsforsch.* **79**, 98 (1973).
- (10) Wynder, E. L., and Hoffmann, D.: "Experimental Tobacco Carcinogenesis". *Adv. Cancer Res.* **8**, 249 (1964).

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- (11) Wynder, E. L.: "Environmental Carcinogenesis and Cocarcinogenesis". Presented in Pharmacol. Symp. on "Mechanisms of Chemical Oncogenesis": 57th Federation Meet., Atlantic City, 1973, April 17.
- (12) Wynder, E. L., and Hoffmann, D.: "Experimental Tobacco Carcinogenesis". *Science* 162, 862 (1968).
- (13) Argus, M. F., and Hoch-Ligeti, C.: "Comparative Study of the Carcinogenic Activity of Nitrosamines". *J. Natl. Cancer Inst.* 27, 695 (1961).
- (14) Smuckler, E. A., Arrhenius, E., and Hultin, T.: "Alterations in Microsomal Electron Transport, Oxidative N-Demethylation and Azo-Dye Cleavage in Carbon Tetrachloride and Dimethylnitrosamine Induced Liver Injury". *Biochem. J.* 103, 55 (1967).
- (15) Thomas, P. E., Kouri, R. E., and Hulton, J. J.: "The Genetics of Aryl Hydrocarbon Hydroxylase Induction in Mice: A Single Gene Difference between C57BL/6 and DBA/2J". *Biochem. Genet.* 6, 157 (1972).
- (16) Nebert, D. W., Goujon, F. M., and Gielen, J. E.: "Aryl Hydrocarbon Hydroxylase Induction by Polycyclic Hydrocarbons: Simple Autosomal Dominant Trait in the Mouse". *Nature (New Biol.)* 236, 107 (1972).
- (17) Kouri, R. E., Salerno, R. A., and Whitmire, C. E.: "Relationships between Aryl Hydrocarbon Hydroxylase Inducibility and Sensitivity to Chemically Induced Subcutaneous Sarcomas in Various Strains of Mice". *J. Natl. Cancer Inst.* 50, 363 (1973).
- (18) Kouri, R. E., Ratrie, H., and Whitmire, C. E.: "Evidence of a Genetic Relationship between Susceptibility to 3-Methylcholanthrene-Induced Subcutaneous Tumors and Inducibility of Aryl Hydrocarbon Hydroxylase". *J. Natl. Cancer Inst.* 51, 197 (1973).
- (19) Kunz, W., Schaudé, G., and Thomas, C.: "Die Beeinflussung der Nitrosamincarcinogenese durch Phenobarbital und Halogenkohlenwasserstoffe". *Z. Krebsforsch.* 72, 291 (1969).
- (20) Hadjiolov, D.: "The Inhibition of Dimethylnitrosamine Carcinogenesis in Rat Liver by Aminoacetonitrile". *Z. Krebsforsch.* 76, 91 (1971).
- (21) Wattenberg, L. W., and Leong, J. L.: "Inhibition of the Carcinogenic Action 7,12-Dimethylbenz[a]anthracene by β -naphthoflavone". *Proc. Soc. Exp. Biol. Med.* 128, 940 (1968).
- (22) Wattenberg, L. W., and Leong, J. L.: "Inhibition of the Carcinogenic Action of Benzo[a]pyrene by Flavones". *Cancer Res.* 30, 1922 (1970).
- (23) Wattenberg, L. W., Leong, J. L., and Strand, P. J.: "Benzpyrene Hydroxylase Activity in the Gastrointestinal Tract". *Cancer Res.* 22, 1120 (1962).
- (24) Conney, A. H. and Levin, W.: "Induction of Hepatic 7,12-Dimethylbenz(a)anthracene Metabolism by Polycyclic Aromatic Hydrocarbons and Aromatic Azo Derivatives". *Life Sciences* 5, 465 (1966).
- (25) Cantrell, E., and Bresnick, E.: "Evidence for Type II Induction of Microsomal Enzymes by β -Naphthoflavone in Rat Liver Preparations". *Life Sci.* 10 (Part II) 1195 (1971).

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

The Seamen's Memorial Research Laboratory at the U. S. Public Health Service Hospital has a total laboratory area of 4800 square feet, plus air-conditioned animal quarters of a total of 3000 square feet. The applicant has at his disposal approximately 1400 square feet of laboratory space, including a well-equipped cold room, approximately 2400 square feet of animal quarter space, and adequate office space. This is made available to the applicant through the Tulane Department of Medicine.

The major equipment in the applicant's laboratory space consists of the following: a Beckman DB spectrophotometer with recorder, a Shimadzu spectrophotometer with flow dichroism attachment, 4 Coleman spectrophotometers, a Photovolt fluorimeter, a Brice-Phoenix light-scattering photometer complete with ratio recorder, an Abbe refractometer, a Rudolph manual spectropolarimeter, 2 fourteen-manometer Warburg apparatuses, a "Yellow Springs" recording oxygen monitor, a high wattage ultrasonic generator, two analytical balances, several pH meters, a semi-automatic tensiometer, a "Spinco L" preparatory ultracentrifuge (including swinging bucket rotor and other accessories for polysome profiling), 3 "Servall" high-speed table centrifuges, 2 heavy-duty International model V centrifuges, a starch-block electrophoresis apparatus with circulating cooling system, a lyophilizing apparatus, 2 heating and cooling precision waterbaths, 2 Dubnoff waterbath incubator shakers, refrigerators, a deep freeze unit, an incubator. The use of a Microtech gas chromatograph and of an osmometer are available in the laboratory.

11. Additional facilities required:

12. Biographical sketches of investigator(s) and other professional personnel (append):

Curricula of three investigators was included with the original application 7/9/73.

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

List of 10 publications see next page.

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13. Publications: (reprints were enclosed with original application 7/9/73).
- (1) Argus, M. F., Sohal, R. S., Bryant, G. M., Hoch-Ligeti, C., and Arcos, J. C.: "Dose-Response and Ultrastructural Alterations in Dioxane Carcinogenesis. Influence of Methylcholanthrene on Acute Toxicity". *Europ. J. Cancer* 9, 237 (1973).
 - (2) Argus, M. F., Valle, R. T., Venkatesan, N., Buu-Hoi, N. P., and Arcos, J. C.: "Molecular-Size-Dependent Effects of Polynuclear Hydrocarbons on Mixed-Function Oxidases: Possible Action on Cascade-Coupled Operons". *Proc. 1st Europ. Biophys. Congr.* EI/38, 187 (1971).
 - (3) Argus, M. F., White, L. E., Bryant, G. M., Arcos, J. C., and Hoch-Ligeti, C.: "Molecular Specificity of the Tumorigenic Action of Ethionine: The Inactivity of S-Ethylcysteine. Action on Respiratory Parameters". *Z. Krebsforsch.* 75, 201 (1971).
 - (4) Arcos, J. C., Venkatesan, N., and Argus, M. F.: "Modification of the Flow Dichroism Spectrum of Rat Liver Nuclear DNA by *In Vivo* Alkylation with Hepatocarcinogenic Dialkylnitrosamines". *Gann* 62, 523 (1971).
 - (5) Venkatesan, N., Argus, M. F., and Arcos, J. C.: "Mechanism of 3-Methylcholanthrene-induced Inhibition of Dimethylnitrosamine Demethylase in Rat Liver". *Cancer Res.* 30, 2556 (1970).
 - (6) Hoch-Ligeti, C., Argus, M. F., and Arcos, J. C.: "Induction of Carcinomas in the Nasal Cavity of Rats by Dioxane". *Brit. J. Cancer* 24, 164 (1970).
 - (7) Arcos, J. C., Mathison, J. B., Tison, M. J., and Mouledoux, A. M.: "Effect of Feeding Amino Azo Dyes on Mitochondrial Swelling and Contraction. Kinetic Evidence for Deletion of Membrane Regulatory Sites". *Cancer Res.* 29, 1288 (1969).
 - (8) Argus, M. F., Walder, J. A., Fabian, J. A., and Arcos, J. C.: "A Study of Soluble Protein and Sulfhydryl Levels in the Rat Liver During Rapid Normal and Premalignant Growth". *Brit. J. Cancer* 22, 330 (1968).
 - (9) Hoch-Ligeti, C., Argus, M. F., and Arcos, J. C.: "Combined Carcinogenic Effects of Dimethylnitrosamine and 3-Methylcholanthrene in the Rat". *J. Natl. Cancer Inst.* 40, 535 (1968).
 - (10) Arcos, J. C., and Simon, J.: "Effect of 4'-Substituents on the Carcinogenic Activity of 4-Aminoazobenzene Derivatives". *Arzneim.-Forsch. (Drug Res.)* 12, 270 (1962).

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10.

14. First year budget:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s)
even if no salary requested)

% time

Amount

Joseph C. Arcos, D. Sc.

20%

Mary F. Argus, Ph. D.

40%

Joseph Simon, D. V. M., Ph. D.

10%

\$ 7,502

Technical

Georgia M. Bryant, B. A., Technician V

50%

4,789

Laboratory Technician (to be recruited)

100%

7,367

Laboratory Technician (to be recruited)

100%

7,367

William Hayes, Senior Animal Attendant

33%

2,215

Assistant Animal Attendant (to be recruited)

100%

4,466

Sub-Total for A

33,706

B. Consumable supplies (by major categories)

Animals - - - - - \$ 5,200

Animal feed - - - - - 2,000

Carcinogens & other Chemical Agents - - - - - 2,000

Glassware - - - - - 500

Animal Bedding & cleaning supplies for animal quarters - 1,200

Sub-Total for B

10,900

C. Other expenses (itemize)

Maintenance Contract for Spinco Ultracentrifuge 350

Contribution to maintenance of cold laboratory 400

Contribution to Xerox rental 200

Sub-Total for C

950

Running Total of A + B + C

45,556

D. Permanent equipment (itemize)

Stereoscopic microscope 800

Animal cages* 2,000

Sub-Total for D

2,800

E. Indirect costs (15% of A+B+C)

E

6,833

Total request

55,189

15. Estimated future requirements: (* and † see next page)

	Salaries	Consumable Suppl.	Other Expenses †	Permanent Equip.*	Indirect Costs	Total
Year 2	35,728	9,600	13,350	5,200	8,802	72,680
Year 3	37,872	7,500	13,350		8,808	67,530

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Explanation of specific budgetary items:

* The species in essentially all investigations in this laboratory in the last 12 years was the rat. Guinea pigs have been used to a small extent. Mice, however, have not been used since 1962. For this reason we have very few cages for mice. Consequently, the funds requested in the first year is for the purchase of a sufficient number of mouse cages to begin the experiments. The funds under "Permanent Equipment" in the second year is partly for the purchase of some additional cages, since the second year is the time of maximum occupancy of the facilities for these experiments, when both the enzyme determinations during carcinogen administration as well as the carcinogenesis experiments will be at their peak. The greatest portion of the funds (\$4,600) for "Permanent Equipment" in the second year is, however, for the purchase of an inexpensive Beckman DB spectrophotometer. This is an essential expenditure, since the two spectrophotometers we now have (a Beckman DB double-beam and a Shimadzu QV-50 single beam) are extensively tied up with ongoing experiments. Moreover, our Beckman DB is a 13 year-old instrument requiring servicing more and more often, which means substantial interruptions in the work. — We computed that the serial enzyme determinations will require the use of a spectrophotometer 60-80% of the time for at least $1\frac{1}{2}$ years, considering the number of groups and the experiments to be carried out in statistically analyzable numbers.

† The category "Other Expenses" in the second and third year represents mainly the expenditures for the histopathological examination of the slides resulting from the carcinogenesis studies. The sums in this category also include the standard yearly expenditures for the items listed in "Other expenses" in the first year.

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16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Faculty Research Award (exclusively salary grant for Joseph C. Arcos)	American Cancer Society	127,887	1/1/72 - 12/31/76
Pharmacology of Repressible Mixed-Function Oxidases	NCI CA-13206	111,626	1/1/72 - 12/31/74

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Pharmacology & Biochemistry of the Oncogen, Dioxane	NCI CA-15111 (NIEHS is also considering funding this application)	384,390	9/1/73 - 8/31/78 (not yet activated as of 1/1/74)
Molecular Bases of the Oncogenic Action of Dioxane	American Cancer Society	139,426	7/1/74 - 6/30/77

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal Investigators:

Date of

Signatures: 1/17/74Typed Name Joseph C. Arcos and Mary F. ArgusSignature Joseph C. Arcos Mary F. ArgusTelephone (504) 899-3441 331

Area Code Number Extension

Checks payable to

Tulane University

Mailing address for checks

Jesse B. Morgan, ComptrollerTulane University, Station 20New Orleans, La. 70118

Responsible officer of institution

Typed Name William G. Thurman, M.D.Title Dean, Tulane Univ. School of MedicineSignature William G. Thurman 1/7/74Telephone (504) 588-5462

Area Code Number Extension

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#972 - FRIEDMAN

1003545121

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

February 7, 1974

Grant application No. 972

CANCER

To: The committee comprising Drs. Gardner, Huebner and Meier

Subject: Marvin A. Friedman, S.B., S.M., Ph.D., Virginia Commonwealth Univ.
New application No. 972
"Suppression of Dimethylnitrosamine and 3-Methylcholanthrene
Carcinogenicity by Nitrogen Dioxide"

History

This applicant initially approached CTR via Dr. Kreisher, who states in a memo dated January 4, 1974 that the proposal was referred "by an industry scientist".

Request

Application No. 972 requests \$39,201 (arithmetic corrected by CTR) plus two additional years, at \$48,000 and \$49,000.

Documents Submitted (attached)

1. Application dated 1-31-74.
2. Biographical sketches of Drs. Friedman, Munson, Elzay, Egle and Chesney.

FWN:gh

Enclosures

FWN
F.W.N.

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972

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

FEB 1 1974

Date: 1-31-74

1. Principal Investigator (give title and degrees):

Marvin A. Friedman, Assistant Professor of Pharmacology
S.B., S.M., Ph.D.

2. Institution & address:

Department of Pharmacology
Medical College of Virginia
Health Science Division
Virginia Commonwealth University
Richmond, Virginia 23298

3. Department(s) where research will be done or collaboration provided:

Department of Pharmacology

4. Short title of study:

Suppression of Dimethylnitrosamine and 3-Methylcholanthrene Carcinogenicity
by Nitrogen Dioxide

5. Proposed starting date:

July 1, 1974

6. Estimated time to complete:

June 30, 1977

7. Brief description of specific research aims:

1. Investigate the correlation between atmospheric NO_2 levels and methemoglobin levels in mice.
2. Quantitate hepatic and pulmonary mixed function oxidase activity subsequent to NO_2 exposure.
3. Determine the kinetics of the effects of NO_2 on mixed function oxidase activity and the effects of NO_2 on cytochrome P-450.
4. Determine the effects of NO_2 on liver, lung, and renal DMN demethylase activity and liver, intestine, lung, and skin aryl-hydrocarbon hydroxylase activity.
5. Investigate the effects of NO_2 on mutagenicity of DMN on the host mediated assay and 3-MC in the dominant lethal test.
6. Determine the effects of NO_2 on transformation of lung cells in vitro by 3-MC or transformation of hamster embryo cells by 3-MC in vitro and by 3-MC and DMN in the host-mediated carcinogenesis assay.
7. Determine the effects of NO_2 on lung and systemic carcinogenic responses to 3-MC and DMN.
8. Determine the effects of NO_2 on 3-MC applied by skin painting.

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Since NO_2 induces methemoglobinemia in experimental animals, we feel that it will have some biochemical properties in common with sodium nitrite, a compound which induces methemoglobinemia in an identical fashion. The particular biochemical lesion in question is the inhibition of liver mixed-function oxidase activity. Our working hypothesis is, therefore, that NO_2 will inhibit mixed function oxidase activity. The direct corollary of this is that NO_2 will also suppress DMN demethylase and aryl-hydrocarbon hydroxylase activities both of which are mixed-function oxidases and both of which activate carcinogens to their proximate form. The end result of this will be protection from mutagenic and carcinogenic effects of dimethylnitrosamine and 3-methylcholanthrene. We, therefore, feel that this component of side-strain cigarette smoke will protect people from the carcinogenic effects of food contaminants and smoke components.

9. Details of experimental design and procedures (append extra pages as necessary)

It is the purpose of this research to evaluate the effects of long term exposure to NO_2 other than the pulmonary toxicity which results from direct action of nitric acid on the lung. The basis for anticipating a systemic response is that NO_2 induces methemoglobinemia. We have shown that sodium nitrite, which induces methemoglobinemia in an identical fashion as NO_2 , suppresses mixed function oxidase activity. Although this induction of methemoglobinemia by NO_2 is well established, there are no published dose-response data for this phenomenon. Therefore, it is important to establish dose-response relationships between atmospheric NO_2 concentrations and methemoglobin levels. With this relationship established, we will be able to better interpret experimental data, and we could better establish whether subclinical methemoglobinemia is a public health concern. Subacute and chronic levels of methemoglobin may then be related in a quantitative fashion to atmospheric NO_2 levels. These dose-response relationships will also be important for setting up protocols for later experiments.

Once methemoglobin levels are established, we will test for methemoglobin mediated suppression of hepatic mixed function oxidase activity. NO_2 would be expected to inhibit liver enzyme activity in a fashion identical to sodium nitrite. Typical Type I and Type II enzymes-namely, aminopyrine demethylase and aniline hydroxylase, will be studied. Enzyme activity will be determined in lung as well as liver because lung is the important site for neoplastic transformation. Since there is a direct effect of NO_2 on lung tissue, one would expect the lung enzymes to be more accessible as well as more severely effected.

In order to study the chronic effects of NO_2 , maximally tolerated levels must be established in a two month subacute experiment. Groups of animals will be exposed to NO_2 for two months and gross pathology taken to determine what doses will be used in later experiments. Once the levels of exposure are determined, the carcinogenicity studies will begin. DMN will be fed to mice who are exposed to varying levels of NO_2 . DMN will be used in these experiments because it induces lung tumors. In addition, DMN induces liver tumors. Therefore, both the local effects of NO_2 on DMN oncogenesis in lung and the systemic effect of NO_2 on DMN oncogenesis in liver can be studied. In addition, DMN is synthesized in vivo from sodium nitrite and secondary or tertiary amines. It is thus apparent that the same populations of people living in industrialized cities who are exposed to high levels of NO_2 also consume preserved and stored foods which contain sodium nitrite or DMN.

(Continued)

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In order to support conclusions drawn from interpretation of DMN data, the oncogenic response to another common pollutant must be studied. We chose 3-MC for many of the same reasons we selected DMN. It induces systemic tumors as well as lung tumors. There is a relationship between levels of enzymes which metabolize it and neoplastic response. It is commonly occurring although more often associated with SO_2 . However, automobile exhaust, the source of most urban NO_2 , is also the source of a considerable amount of 3-MC. In addition, 3-MC is a human carcinogen so these observations are relevant to human exposure. 3-MC has topical activity as well as systemic activity. We can, therefore, determine whether NO_2 modifies epidermal responsiveness to polycyclic hydrocarbons.

There are apparent problems in interpretation of the in vivo reactions. The susceptibility of the animal to infection will be increased, appetite modified, behavior changes, and so forth. Therefore, it is important to support our conclusions by in vitro observations. We will expose lungs in vitro to polycyclic hydrocarbons either alone or in combination with varying levels of NO_2 . This system correlates very well with in vivo responses in the sense that agents which are active in the lungs in vitro are also active in vivo and lungs from strains of mice which are resistant to lung tumors are also resistant in vitro.

We will also test for interactive responses between NO_2 and polycyclic hydrocarbons in trypsinized hamster embryo cells in primary or secondary culture. Although this system is not as conceptually related to the in vivo system, there are many similar metabolic considerations, and it is predictive in predicting carcinogenic potential of polycyclic hydrocarbons. We will bridge the gap between these studies and the in vivo studies by determining the effects of NO_2 on DMN and 3-MC transformation in the host-mediated carcinogenesis test.

It is noteworthy that we have not indicated that we will test DMN in vitro. There is no background literature indicating that DMN is active in vitro carcinogenicity tests. We will of course test DMN, both alone and in combination, but the basis for instigating these tests is not as sound as in the case of polycyclic hydrocarbons. However, we will test DMN in the host-mediated carcinogenicity test for which data indicates that DMN is positive. Due to the rapidity of the responses, the low cost of the experiments and ease of performing these experiments, other carcinogens may be tested in vitro or in the host-mediated carcinogenicity test for synergy or antagonism with NO_2 . In addition, other gaseous air pollutants such as SO_2 , SO_3 , or CO may also be tested. These are inexpensive experiments which will provide a great amount of information.

It has been our opinion that mutagenic potential of environmental pollutants may represent as great a potential public health hazard as the carcinogenic potential. Therefore, we will also evaluate the interactive effects of NO_2 on DMN and 3-MC mutagenicity. One of the problems in these mutagenicity experiments is that DMN is a potent mutagen in the host-mediated assay but inactive in the dominant lethal test while 3-MC is inactive in the host-mediated assay but active in the dominant lethal test. We will therefore, set up and perform the relevant test for each compound. There are other advantages to these tests. In the case of the host-mediated assay, we can determine whether NO_2 will reach in body fluids to produce mutagenic nitrosamines. As we indicated

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earlier, this is a sensitive system, detecting inhibition of DMN mutagenicity at doses as low as 16.5 mg/kg sodium nitrite. In the case of the dominant lethal test, we will be able to determine if NO_2 has reproductive toxicity. We will routinely measure fertility index as part of our dominant lethal test. If it appears that NO_2 has reproductive toxicity, we will then perform a three generation reproduction test to quantitate the magnitude of the response.

Finally, we must establish directly the mechanism of the responses to NO_2 . We will measure P-450 levels and spectra to determine how NO_2 is modifying mixed function oxidase activity. We will look not only for P-450 levels but also for characteristic nitroso groups which will indicate that NO_2 nitrosates P-450. We will also determine the direct effects of NO_2 on the enzyme which metabolize these carcinogens. In the case of DMN, DMN demethylase will be assayed from liver, lung, and kidney because these are the sites of neoplastic transformation. This enzyme will be measured in vitro by assay of formaldehyde produced from incubation of liver microsomes with DMN in the presence of relevant cofactors or alkylation of protein and nucleic acids by DMN- C^{14} . Analogous experiments will be performed to determine the effects of NO_2 on aryl-hydrocarbon hydroxylase. In vitro studies to determine the aryl-hydrocarbon hydroxylase activity in the liver, intestine, lung, mammary gland, and skin will be determined. Intestine, lung, mammary gland, and skin will be studied because these are sites of neoplastic transformation. Liver will be studied because it is the site of catabolism of polycyclic hydrocarbons prior to excretion. For example, inhibition of liver enzyme activity will increase the 3-MC half life in the animal and increase the possibility of neoplastic transformation. Increases in liver enzyme activity by DDT have been shown to decrease the oncogenicity of 3-MC.

The strain of mouse to be studied is very important to interpretation of the experiments. We have a broad spectrum to choose from ranging from C57BL which is markedly resistant to lung tumors to the Strain A mouse which is sensitive to lung tumors to the AKR which carries a leukemia virus activated by 3-MC. The two strains of mice we have most experience with are ideal for these studies because they are sensitive to the lung carcinogenicity of 3-MC and DMN and are hearty, strong animals which can tolerate the air pollutants. We routinely use Swiss mice because of cost consideration, health considerations, (they are an infection-resistant mouse) and extrapolation of data to a random bred human population. They are random bred mice so we feel they are most characteristic of the population responses, and the data is more relevant for humans. This strain will be the mouse used in the bulk of the studies. We often need an inbred mouse for problems of tumor biology, and we have selected the C3H/HE. This is a good inbred strain which is sensitive to lung carcinogenicity by 3-MC. This strain does not carry the Bittner factor for spontaneous mammary tumors. These animals will be used for in vitro tests and to confirm the results obtained in studies on Swiss mice. There will also be occasion to study a resistant strain and in these studies C57BL mice will be used.

It will also be essential to have accurate data on urban levels of NO_2 so that the dose response data obtained here can be interpreted in view of community health. This data will be obtained by the Mobile Unit at the Richmond Air Pollution Control Center. With experimental dose-response data and accurate measurements of the levels of pollutants in urban areas-namely, Richmond-we can evaluate the significance of ambient NO_2 levels.

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METHODS OF PROCEDURE

1. Animals

Male Swiss (ICR) mice will be used unless otherwise stated and are available by direct truck from Flow Laboratories in Dublin, Virginia. C3H/HE mice will be used in some experiments and also are available from Flow Laboratories. C57BL mice when required will be obtained from Sprague Dawley in Madison, Wisconsin. Mice will be housed in shoebox-type plastic cages covered by disposable filters. Mice will be maintained on agar gel diets containing the carcinogens (1). We have found agar gel diets are a very safe and efficient way to handle toxic diets. There is a sufficient variation in stock diets that a semipurified diet is almost essential for interpretation of long-term experiments.

There will be two types of inhalation chambers used in these studies. The geometrics of the exposure system is not a matter of great concern since NO_2 is a freely diffusible gas and does not settle and cannot be filtered through fur. For acute studies, systems resembling the cylindrical glass battery as described by Leach and for chronic studies, chambers similar to the New York University model will be used (2). The cylindrical-type cages, 4" in diameter and 12" long, can be readily constructed at the Medical College of Virginia and will be ready at the point of initiation of these studies. For chronic experiments, the design is more complex. A plexiglass box, 3' by 3', opening on one side and with three shelves will be constructed here at M.C.V. The bottom and top will be constructed as pyramids to insure an even air flow. The smaller cages can be used for the subacute experiments, and no time will be lost in construction of these cages. We have at the Medical College of Virginia necessary plastic cages and accessories for the rest of the study.

2. Environmental NO_2 levels:

The mobile lab at the Richmond Air Pollution Control Center is currently set up to measure ambient NO_2 levels by established E.P.A. procedures (3). In collaboration with this project, the mobile unit will be stationed at high traffic areas in the City of Richmond to measure NO_2 levels. Additionally, levels of exposure of experimental animals will also be constantly monitored by procedures we find are better suited for routine laboratory analyses (4).

3. Microsomal Mixed Function Oxidase Determinations and Methemoglobin Assays

Methemoglobin levels will be determined on peripheral blood isolated from the tail. The percentage of methemoglobin will be determined by the difference in absorbancy of blood before and after addition of sodium cyanide (5). Both the time course and the dose response to NO_2 will be determined.

Mouse liver, kidney, and lung mixed function oxidase activities will be determined following chronic and acute exposure to NO_2 . Aniline hydroxylase and aminopyrine demethylase activities will be determined.

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Assays for drug metabolizing enzyme activity will be performed on crude microsomes. Mice will be killed by decapitation, and the test organs will be removed and chilled. Following homogenization in sucrose buffer, samples will be centrifuged at 10,000 x g for 15 minutes. Preparations isolated in this manner are then stable to freezing, if necessary. Determinations of the activity of several different enzymes can be performed on the same microsomal enzyme preparation.

The standard incubation mixture we have employed in previous experiments with sodium nitrite contained 75 u moles nicotinamide, 4.5 u moles NADP, 15 u moles $MgCl_2$, 30 u moles $MnCl_2$, 15 u moles D, L isocitrate, and 75 gm isocitric dehydrogenase and 0.3 ml of microsomal preparation. Following incubation at 37° in a Dubnoff shaker, the reactions are stopped and enzyme activity quantitated. Microsomal aniline hydroxylase activity is quantitated by measuring p-aminophenol production while aminopyrine demethylase is assayed by determining formaldehyde production (6).

The dose-response of liver and lung mixed function oxidase activities to single 45 minute exposure to varying levels of NO_2 from 50 ppm to 0.4 ppm will be determined. Similarly, the time course of onset of effects from a single 45 minute exposure to 50 ppm NO_2 as well as the duration of effects from the exposure will also be determined. Subacute studies will then be performed to test whether these effects are cumulative.

In subsequent experiments the effects of NO_2 on K_m and V_m of lung and liver mixed function oxidases will be determined at times of maximum suppression of enzyme activity (6). These data will indicate whether the inhibitory effects of NO_2 are competitive, uncompetitive, or noncompetitive. Then cytochrome P-450 levels and spectra will be determined (7) in order to establish whether the observed effects are mediated through cytochrome P-450.

4. Aryl-Hydrocarbon Hydroxylase and DMN Demethylase Determinations

The acute, subacute, and chronic effects of NO_2 exposure on aryl-hydrocarbon hydroxylase and DMN demethylase activities will be determined. Aryl-hydrocarbon hydroxylase will be measured by determining conversion of benzpyrene to 3-hydroxybenzpyrene (8). In some studies 3-MC-6-C¹⁴ will be added and its metabolic products identified. DMN demethylase will be assayed by quantitating the methylation of protein and nucleic acids following incubation with DMN-C¹⁴ (9). We will also perform formaldehyde determinations to measure the enzyme activity. In all cases the incubation medium and enzyme sources described for aminopyrine demethylase and aniline hydroxylase will be used for these studies.

Acute, subacute, and chronic studies of the effects of NO_2 on aryl-hydrocarbon hydroxylase and DMN demethylase will be performed. Enzyme assays will be performed at periods when aminopyrine demethylase and aniline hydroxylase are suppressed. Enzyme assays will be performed following single exposure to NO_2 and at monthly intervals in experiments under conditions identical to those in the long term studies. In the case of aryl-hydrocarbon hydroxylase, enzyme activity in intestine, liver, lung, and skin will be measured (10). In the case of DMN demethylase, liver, kidney, and lung enzyme activity will be measured.

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5. Mutagenicity Testing

We will test for reverse mutation of S. typhimurium G-46 in a host mediated assay (11). This system has shown that sodium nitrite antagonizes DMN mutagenicity (12). Groups of 10 mice will be injected I.P. with S. typhimurium G-46 and sacrificed three hours later. The organisms will be recovered, diluted appropriately, and plated. All animals will be run individually.

Mice will be exposed to NO_2 in either acute dose-response or chronic experiments. Then each animal will be treated I.M. with DMN and I.P. with bacteria. Relevant solvent and unexposed mouse controls will be performed. Mutant frequency in recovered S. typhimurium will be determined.

We will also determine the effects of NO_2 in the dominant lethal test (13). Groups of 10 male mice will be exposed either acutely or chronically to NO_2 and mated with two virgin females per male each week for eight weeks. Females will be sacrificed 14 days after initiation of mating and number of pregnancies, implantation sites, and early fetal deaths recorded. Groups of mice will be treated with 3-MC either alone or in combination with NO_2 .

6. In Vitro Carcinogenicity Tests

Interactions between NO_2 and 3-MC or DMN will be tested in vitro in two test systems. In one system (14), 12-14 day old fetuses of randombred Syrian hamsters will be grown in Eagle's minimum essential medium with 10% calf serum and exposed to 3-MC (5 ug/ml) in the dark. Cells will be plated in complete medium with 10% bovine serum in a petri dish containing 6×10^4 irradiated rat embryo cells. Hamster cells will be seeded on cover slips adjusted to cover most of the surface of the petri dish. Nine days after addition of 3-MC, the dishes will be examined with phase microscopy for transformed colonies. Some plates will be fixed and stained with Giemsa for further analysis. All incubations will take place under controlled relative humidity, 10% CO_2 , 37°C and varying concentrations of NO_2 . The pH will be monitored at all times since NO_2 is acidic.

The synergistic or antagonist carcinogenic potential of NO_2 will also be evaluated in the host-mediated in vivo - in vitro assay for chemical carcinogenesis (15). Pregnant Syrian golden hamsters at 10 to 11 days gestation will be injected I.P. with 5-30 mg/kg DMN or 100-300 mg/kg 3-MC. These animals will be exposed to NO_2 either acutely in time-response experiments or in dose-response experiments or chronically for 1-6 months prior to mating. The hamsters will be killed 48-72 hours after treatment (on Day 13 of gestation), and the fetal cells cultured in Eagle's medium with 10% fetal bovine serum. These cells will be passed in a ratio of 1:10 every 4-6 days for at least three passages.

In a second series of experiments, lungs from one month C3H/He mice will be exposed in vitro to 3-MC and implanted into mice of the same strain (16). Whole lungs from one month old mice will be cut into pieces approximately $2 \times 1 \times 1$ mm and cultured on strips of cellulose acetate in petri dishes containing Trowell's T8 culture media with 15% added C3H serum. Solutions of 3-MC in acetone will be added to the medium to give final concentrations of approximately 5 ug/ml. Explants will be cultured for one day in control medium, four days

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9. Continued

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on carcinogen containing medium, and one day in control medium. These explants will then be implanted subcutaneously in six week old C3H/He mice and the animals observed for up to 12 months. The variables tested in these experiments involve: 1) The effects of NO_2 in vitro on 3-MC transformation of lung cells, and 2) The sensitivity to in vitro transformation of lung cells from animals previously exposed to NO_2 .

7. Lifetime Carcinogenicity Studies

The following groups and numbers of mice will be exposed for their entire lifetime to NO_2 either alone or in combination with a carcinogen.

<u>Group</u>	<u>NO_2 Exposure</u>	<u>Carcinogen Exposure</u>
I	none	none
II	high dose ca 3 ppm	none
III	low dose ca 0.5 ppm	none
IV	none	DMN in diet
V	high dose	DMN in diet
VI	low dose	DMN in diet
VII	none	3-MC in diet
VIII	high dose	3-MC in diet
IX	low dose	3-MC in diet
X	none	3-MC topically
XI	high dose	3-MC topically
XII	low dose	3-MC topically

In the case of Groups X, XI, and XII where induction of skin tumors is one of the end points, some experimental conclusions may come as early as six months allowing dose-response experiments to be performed as repeats. Doses of 3-MC will be applied in acetone solution to the backs of shaved mice. Mice will then be shaved at weekly intervals and inspected for papillomas.

Doses of NO_2 will be determined in two month preliminary dose response experiments. The high dose used will be the maximally tolerated dose (i.e. the dose below which no weight loss occurs). The low dose will be 1/10 the maximally tolerated dose. DMN will be fed in agar diets at a level of 5 ppm which gives a spectrum of tumors in mice (17), and 3-MC will be fed at 10 ppm which also gives a broad spectrum of tumors in mice (18). Mice will be inspected twice daily weekdays and on weekends for tumors or morbidity or mortality. Dead or morbid animals will be autopsied and liver, spleen, lung, and G.I. tract taken for histopathology. Body weights will be recorded weekly for the first six weeks and then monthly starting at eight weeks. Mice will be exposed to NO_2 for eight hours daily, seven days per week. The limited exposure is to insure that the food and carcinogens in the food are not effected by NO_2 .

8. Potential Interactions Among Other Gases

In the latter stages of the project, when we have exposure chambers standardized, and most of the data on NO_2 , we will test other gases for acute effects. We will study CO and SO_2 primarily either alone or in combination with each other or NO_2 . We will also investigate HCl either alone or in combination with NO_2 . We will test these gases for effects on liver and lung

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DMN demethylase and aryl-hydrocarbon hydroxylase and on transformation of fetal hamster cells by DMN or 3-MC in the host-mediated carcinogenesis system. It is important to note that in these experiments as well as all previous experiments, non-carcinogen treated controls will be performed so that we will have an accurate index of the biochemical effects of these gases alone or in combination with each other.

References

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2. Described by Drew, R.T., and Lasken, S., in *Methods in Animal Experimentation*.
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12. Couch, D.B., McClanahan, H., and Friedman, M.A., *Fed. Proc.* 32:833, 1973.
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16. Flaks, A., *Eur. J. Cancer*, 6:259 (1970).
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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Facilities Available

Laboratory facilities sufficient for the staff described here are available in McGuire Hall. The Pharmacology Department has sufficient animal quarters to allow a complete room for the sole use of mice in the carcinogenicity and mutagenicity studies described here. Another room will be used solely to house the inhalation exposure chambers. Available instruments include a Varian UV-visible double beam spectrophotometer, Packard Scintillation Counter, International Model J centrifuge, L-3 ultracentrifuge, Beckman gradient former, Isco gradient scanner, Labconco glassware washer, and others solely responsible to Dr. Friedman. Additionally, the Department of Pharmacology has five gas chromatographs, another ultracentrifuge, a Packard Oxidizer, two other scintillation counters, several fluorometers, I-R and N.M.R. spectrophotometers, mass spectrometer, CO₂ incubators, autoclave, tissue culture hoods, inverted scope, and a room solely devoted to tissue culture.

All carcinogenic material including DMN and 3-MC will be prepared in a hazardous substance room. This room is equipped with a glove box and filtered exhaust fan. All personnel on this project will go through a training program to learn technique in handling these hazardous substances. This training program will be conducted by the principal investigator and Dr. Joseph F. Borzelleca who was Chairman of Standards Committee on Carcinogens for the Occupational Safety and Health Administration for the Department of Labor.

(Continued)

11. Additional facilities required:

Dr. Friedman is director of the carcinogenesis program area of MCV/VCU Comprehensive Cancer Research Center (CCRC). Many of the individuals discussed here are part of this Program Area. The facilities and complete support of the CCRC will be available for this project.

Tissue and organ culture will be performed by Mrs. J. Munson. She currently maintains our tumor cell lines and determines direct cytotoxicity either with drugs or with lymphocytes and/or macrophages. She also has experience in cell transformation *in vitro* in our studies using Friend leukemia virus. Dr. Cribbs will consult on cell transformation data and mutagenicity experiments. Mr. J. G. Williams, Director of the Richmond Air Pollution Control Center, will collaborate in monitoring the NO₂ exposure levels. Similarly, he will perform relevant environmental surveillance of ambient NO₂ levels.

Histopathology will be performed with the collaboration of several pathologists. Everyday observation of the animals will be performed by Dr. Sawyer. Dr. Chesney, a veterinary pathologist, will be joining our staff soon. Dr. Richard Elzay and Dr. Frank Rea have aided in our histopathology in the past and will consult on this project.

12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

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10. Continued

Studies involving mice will conform with the GUIDE FOR THE CARE AND USE OF LABORATORY ANIMALS prepared by the Institute of Laboratory Animal Resources, National Research Council (DHEN publication no. (NIH) 73-23) and with the federal laws and regulations. The quality of animal care will be scrutinized by the MCV/VCU Animal Care Committee and Dr. Chesney.

1003545133

14. First year budget:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s) even if no salary requested)	% time	Amount
Marvin A. Friedman	30	
Albert E. Munson	30	
John L. Egle, Jr.	30	
Richard P. Elzay	20	
Danny R. Sawyer	100	
Charles F. Chesney	30	

Technical

Judith A. Munson	50	4402
Marilyn Green	50	4402
Kathleen Watt	50	4402
Walter Bullock	100	5943

Sub-Total for A 19149

B. Consumable supplies (by major categories)

Chemicals	2500
Glassware	2000
Animals	3000
Animal Care	1000
Semisynthetic Diets	2000

Sub-Total for B 10500

C. Other expenses (itemize)

Travel	500
Histopathology	1500
Chart Drawings and	
Photography	200
Page Charges & Reprints	300
Telephone	100
Postage	50
Laundry	50

Sub-Total for C 2700
2200

Running Total of A + B + C 32349

D. Permanent equipment (itemize)

Exposure Chambers	2000
-------------------	------

Sub-Total for D 34349

E. Indirect costs (15% of A+B+C)

E 5152

Total request 39501

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	20298	13000	6000	2000	6195	47493
Year 3	21516	13000	6000	2000	6377	48893

1003545134

16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Nitrosamine: An Environmental Hazard	NIEHS: ES00713	98,168	Jan 1, 1972-Dec 31, 1974

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Interactive Effects of of Piperonyl Butoxide	NIEHS: ES00925	89,990	March 1, 1974-Feb 28, 1977

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Checks payable to

er Lossing, Controller

Mailing address for checks

1201 East Broad Street

Richmond, Virginia 23298

Principal investigator

Typed Name Marvin A. Friedman, Ph.D.

Signature Marvin A. Friedman Date 1-31-74

Telephone 804 770 4670
Area Code Number Extension

Responsible officer of institution

Typed Name L. A. Woods, M. D., Ph.D.

Title Vice President for Health Sciences

Signature L. A. Woods Date 1-31-74

Telephone 804 770 4001
Area Code Number Extension

1003545135

#944 - GLICK

1003545136

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

October 22, 1973

Grant Application #944

CANCER

To: The committee comprising Drs. Andervont, Huebner and Meier

Subject: David Glick, Ph.D., Stanford Research Institute, Menlo Park
New application No. 944
"Quantitative Histochemical Studies on Lung Cancer Induced
by Carcinogenic Agents"

History

This proposal was case #228 and formal application was encouraged.

Request

Application #944 requests \$60,573.00 for an initial year. An additional two years' request is stated to be contingent upon outcome of the first year's work.

Document Submitted

Attached is application dated Oct. 1, 1973.

Comment

Page 2, item 9, states that collaboration with Dr. Arthur Furst is proposed. We are obtaining a statement from Dr. Furst in this regard.

FWN:gh

Enclosure

JM

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#944

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

110 EAST 59TH STREET

NEW YORK, N. Y. 10022

(212) 421-8885

OCT 19 1973

Application for Research Grant

Date: Oct. 1, 1973

(Use extra pages as needed)

1. Principal Investigator (give title and degrees): David Glick, Ph.D., Director
Center for Histochemical Research
2. Institution & address: Stanford Research Institute
Life Sciences Division
333 Ravenswood Avenue
Menlo Park, CA 94025
3. Department(s) where research will be done or collaboration provided: Life Sciences Division

4. Short title of study:

Quantitative Histochemical Studies
on Lung Cancer Induced by Carcinogenic
Agents

5. Proposed starting date:

November 1, 1973

6. Estimated time to complete:

1 year. Renewal to be dependent on mutual
desire of both parties.

7. Brief description of specific research aims:

(1) Quantitative determination of cyclic AMP (cAMP) in cytologically defined samples of mouse lung tumors (adenomas) induced by benzpyrene and squamous cell carcinoma induced by 3-methylcholanthrene. Changes in cAMP concentration with correlation to the cytology will be followed as a function of malignant development and compared with appropriate nonmalignant controls. The aim is to clarify to what degree these carcinogens can affect the cAMP control in lung cell function during carcinogenesis.

(2) Parallel study of benzpyrene and 3-methylcholanthrene hydroxylase activity in the same material. This will first require adaptation of the method for determination of the specific hydroxylase activity to the microscale needed for the small samples.

The aim is to clarify to what degree this enzyme, which is responsible for the first step in detoxification and elimination of the carcinogen, may undergo change in the induced carcinogenesis, possibly in an adaptive mechanism for defense against the action of the carcinogens.

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8. Brief statement of working hypothesis:

2.

(1) Involvement of cAMP in cell membranes with malignant development is now established; e.g., it has been shown recently that cAMP suppresses tumorigenicity of human cancer cells (Smith and Handler, Res. Commun. Chem. Pathol. Pharmacol. 5, 863, 1973; Fed. Proc. 32 (3), Pt. 1, Abstr. 2226, 1973), that cAMP levels are lower in cancer cells than in normal cells, and that cAMP is critically involved in cell-to-cell contact effects and their alterations in cancer (Emmelot, European J. Cancer 9, 319, 1973).

Accordingly, it is important to clarify the cAMP role in carcinogenesis in lung as induced by benzpyrene and 3-methylcholanthrene.

(2) Detoxification and excretion of benzpyrene and 3-methylcholanthrene are involved in the physiological defense to the action of the carcinogens; if there were an adaptive increase in the specific hydroxylase acting on these compounds to promote their transformation and elimination, this would oppose their influence in inducing lung tumors. It is essential to know to what degree the lung enzyme is affected by the hydrocarbons and how the enzyme activity changes in carcinogenesis.

9. Details of experimental design and procedures (append extra pages as necessary)

The proposed research will be conducted in collaboration with Dr. Arthur Furst, Institute of Chemical Biology, University of San Francisco, who will provide the tissue from material he is already producing under a grant from the Council for Tobacco Research - U.S.A. Thus, additional information could be obtained from his material without interfering in any way with Dr. Furst's work, since the quantities of tissue needed in the quantitative histochemical studies proposed are very small.

Dr. Furst will provide lung tumor tissue from his genetically selected mice (C57Bl/6/J, C57Bl/6/Cum, dba/J, NIH-Swiss/MBA), in which the hydrocarbon-induced tumors have been formed, and control tissue samples from untreated mice, from nontumor regions of the lungs with tumors, and from corresponding benign lung tumors. He will also provide the histological examination and diagnosis of the tissues used. Each sample will be divided, one part for histological preparation and diagnosis by Robert Kovatch, D.V.M., and the other immediately fresh-frozen and delivered for analysis by microchemical procedures of quantitative histochemistry. Samples will be prepared for measurement by cryostat sectioning of the fresh-frozen material, and the individual microtome sections will be collected in separate small glass tubes for chemical analyses, with an adjacent section to each one used for analysis taken for histological staining and diagnosis.

The cAMP will be measured by the fire-fly luminescence method as adapted by Orenberg and Glick (J. Histochem. Cytochem. 20, 923, 1972) for analysis of microtome sections of tissue, using improvements and simplifications more recently developed in our laboratory.

Depending on which proves most feasible for the present purpose, the specific hydroxylase activity will be measured by an adaptation of the fluorescence method described by Wattenberg et al. (Cancer Res. 28, 934, 1968), Nebert et al. (J. Biol. Chem. 243, 6242, 1968; Cancer Res. 29, 1763, 1969), or Kouri et al. (J. Nat. Cancer Inst. 49, 993, 1972).

Measurement of the amount of sample analyzed for calculation of concentration will be the wet weight (to permit comparison with certain published data) and the protein content (more analytically reliable and also used in certain published data). The latter will be measured by the bromsulphalein-binding method for protein, in use in the laboratory of the principal investigator for many years (Glick, Quantitative Techniques of Histo- and Cytochemistry, Vol. II, pp. 148-150, 155-160, Wiley - Interscience, N.Y., 1963).

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3.

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

A laboratory (644 ft²) is available at Stanford Research Institute, Life Sciences Division (Building 28, Room Nos. 202 and 203), for the quantitative histochemical work.

11. Additional facilities required:

The aspect of the investigation that involves the collaboration of Dr. Furst will be conducted in his laboratory, which also has adequate animal and histology facilities (already site-visited by CTR personnel).

12. Biographical sketches of investigator(s) and other professional personnel (append):

Appendices 1, 2, 3.

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

Appendix 4, plus reprints.

1003545140

14. First year budget:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s) even if no salary requested)	% time	Amount		
		Salaries	Benefits	Total
David Glick, Ph.D., Principal Investigator	25	7,332	1,906	9,238
Dorothy von Redlich, Ph.D., Res. Assoc.	50	8,889	2,311	11,200
Yoshinao Katsumata, M.D., Res. Assoc.	75	10,672	2,775	13,447
Research Asst.-to be recruited	50	3,733	971	4,704

Technical

Secretary, to be recruited	25	1,793	466	2,259
Dishwasher, Lab. Helper	25	1,603	417	2,020
		34,022	8,846	42,868

Sub-Total for A 42,868

B. Consumable supplies (by major categories)

Chemicals		
Lab. Ware		1,200
		<u>800</u>

Sub-Total for B 2,000

C. Other expenses (itemize)

Publications, Drawings, Photos, and Reports	400
Upkeep of Instruments	400
Miscellaneous	500
Travel-equiv. of 1 East Coast trip for 2 investigators	1,200
	<u>2,500</u>

Sub-Total for C 2,500

D. Permanent equipment (itemize)

Luminometer (du Pont)	6,100
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Sub-Total for D 6,100

E. Indirect costs (15% of A+B+C)

E 7,105

F. Estimated future requirements:

Total request 60,573

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	Although anticipated as a 3-year project, the budget for the first year only is					
Year 3	given because future budgets would be determined by requirements made apparent					

1003545141

4a.

15. Estimated future requirements: (Continued)

in the first year. Renewal for subsequent years of the grant to depend on mutual desires of both parties.

1003545142

16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Quantitative Histo- chemistry of Gastric Acid Secretion	NIH - submitted	169,102	5/1/74 - 4/30/77

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Checks payable to

Stanford Research Institute

Mailing address for checks

Life Sciences Business Office
Stanford Research Institute
333 Ravenswood Ave., Menlo Park, CA 94025

Principal investigator

Typed Name David Glick

Signature David Glick Date Oct. 1, 1973

Telephone 415 326-6200 2116
Area Code Number Extension

Responsible officer of institution

Typed Name Philip J. O'Donnell

Title Manager, Contract Administration

Signature Philip J. O'Donnell Date Oct. 15, 1973

Telephone 415 vc 326-6200 2482
Area Code Number Extension

1003545143

#946 HERRMANN

1003545144

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

November 29, 1973

Grant Application No. 946

CANCER

To: The committee comprising Drs. Andervont, Gardner, Meier

Subject: Robert L. Herrmann, Ph.D., Boston University School of Medicine
New application No. 946
"Effect of Carcinogens on Nuclear Phospho proteins and
Phosphorylating Enzymes"

History

This is a spontaneous application, with no known antecedents other than a request from Dr. Herrmann for application forms dated May 2, 1972.

Request

Application No. 946 requests \$11,335 plus two additional years.

Documents Submitted (attached)

1. Application, undated, received by CTR November 12, 1973 (25 pages).
2. Reprints of papers identified as Number 2 and Number 4, page 25 of the application.

Comment

The applicant's remarks on relationships among various pending research grant applications, page 5 of the application, are noteworthy.



F.W.N.

FWN:wg
Encls.

1003545145

CANCER

Comm.

Dr. Andervont
Dr. Gardner
Dr. Meier

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022

NOV 12 1973

Application For Research Grant

Date:

1. Name of Investigator(s): (include Title and Degrees) Robert L. Herrmann, Ph.D.
Associate Professor of Biochemistry

2. Institution & Address: Boston University School of Medicine
80 E. Concord Street, Boston, Mass. 02118

3. Short Title of Project: Effect of Carcinogens On Nuclear Phosphoproteins
and Phosphorylating Enzymes

4. Proposed Starting Date: March 1974

5. Anticipated Duration of this Specific Study: 3 years.

6. Brief Description of Objectives or Specific Aims:

We propose to examine the effects of various carcinogens on nuclear protein phosphorylation.

The steps in this process are planned as follows:

- a.) Isolation and characterization of enzyme systems present in C57BL/6 mouse liver and lung nuclei which phosphorylate nuclear proteins.
- b.) Examination of the effects of various carcinogens on these isolated enzyme systems.
- c.) Comparison with the corresponding enzymatic activities isolated from normal, carcinogen-treated and tumor-bearing mice.

7. Give a Brief Statement of your Working Hypothesis: Recent research has pointed out the
(Cont. on next page)

1003545146

important role of nuclear protein phosphorylation in the regulation of cell division and differentiation. In many animal and cell culture systems there is a co-relation between the functional state of a cell and its patterns of histone and non-histone chromosomal protein (NHCP) phosphorylation. Normally vs. abnormally proliferating cells and differentiated vs. embryonic or dedifferentiated cell types display characteristic differences in this regard. We propose to examine the effects of various carcinogens on nuclear protein phosphorylation with the expectation that these agents may significantly alter the enzyme systems involved. Such alterations may possibly be closely correlated with the establishment of the neoplastic state.

1003545147

8. Details of Experimental Design and Procedures: (Attach Separate Pages)

Our interest in nuclear protein phosphorylation stems from two types of observations, both of which bear on the cancer problem.

- 1) Histones and NECPs each display transient, tissue- and growth-specific patterns of phosphorylation which appear to relate to transcriptive and replicative capability in the case of histones and to degree and quality of gene activation in the case of non-histones.
- 2) Many virus particles possess phosphorylated protein moieties which may play similar roles in cellular, as well as viral replication; and many of these viruses possess endogenous protein kinase activity capable of phosphorylating host cell as well as virion proteins in vitro.

Many published studies substantiate the relationship between chromosomal protein phosphorylation and the rate of cell division. Balhorn reports that in developing rat liver the level of F1 histone phosphorylation decreases from a high of 80% to undetectable levels between 15-day embryonic and adult animals, when the mitotic index has fallen to a few per cent of maximum. (Balhorn et al 1972a) (Cont. on next page.)

9. Physical Facilities Available (Where Other than Administering Organization Indicate Geographical Location)

See Page 23.

10. Additional Requirements: none

11. Biographical sketches of all principal and professional personnel (append)

See Page 24.

12. List of publications: (Five most recent as pertinent) (append)

See Page 25.

1003545148

13. Budget: (1st year)

A. Salaries (Personnel by names)

Professional Robert L. Herrmann,
Principal Investigator

% time

Amount

15

REDACTED

Technical

Technical Assistant, to be
selected

50

Fringe benefits

Sub-Total

REDACTED
REDACTED

B. Consumable Supplies (list by categories)

Radioisotopes
Enzymes, Reagents
Drugs
Glassware

1,000

500

300

800

Sub-Total

2,600

C. Other Expenses (itemize)

Animals - C57BL/6 & BALBc mice
Animal Care

500

400

Sub-Total

900

D. Permanent Equipment (itemize)

Incubator-Shaker - Precision
Replacement Rotor
Servall SS34 for RC2B centrifuge
Fraction Collector Buchler fractomette

850

400

1,000

2,250

1,185

E. Overhead (15% of A+B+C)

Total

11,335

Estimated Future Requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Overhead	Total
Year 2	R	2,000	1,200	1,000	1,230	10,430
Year 3	R	2,200	1,500	800	1,380	11,380

It is understood that the applicant and institutional officers
in applying for a grant have read and found acceptable
the Council's "Statement of Policy Containing Conditions
and Terms Under Which Project Grants Are Made."

Signature

Robert L. Herrmann

Signature

Robert M. Jacob

Telephone

617 262-4200

x6101

Telephone

1003545149

Other Sources of Financial Support

List financial support for research from all sources, including own institution, for this and/or related research projects.

Current	Title of Project	Source	Amount	Duration
	Genetic Recombination in <u>E. coli</u>	National Science Foundation	\$38,000	2 yrs. 12/15/72- 12/14/74
Pending	Transcriptional Diversity in Aging Human Brain	U.S.P.H.S.	92,984	3 yrs. 1/1/74- 12/31/76
	*Nuclear Phosphoproteins and Phosphorylating Enzymes in Neoplasia	U.S.P.H.S.	69,790	3 yrs. 1/1/74- 12/31/76
	** Effect of Carcinogens on Nuclear Phosphoproteins and Phosphorylating Enzymes	Damon Runyon Cancer Fund	19,730	3/1/74- 2/28/75

1003545150

* We have applied to the U. S. Public Health Service for funds to isolate and characterize histone and non-histone proteins from chromatin of human colon and colon carcinoma and from 3T3 cells and their SV-40-transformed counterparts. Some of the purification work outlined in that proposal will follow similar procedures to those proposed here, but there are so many differences involved in using mouse tissues and the in vivo studies with the mouse that we regard the two proposals as essentially distinct.

** The application to the Damon Runyon Fund is essentially a duplicate of the present proposal. However, the breadth of the proposal is so great that we would like to request consideration of at least partial support even if the Damon Runyon grant is funded. This would allow us an earlier start on both invitro and in vivo phases of the investigation and also to place greater emphasis on the tobacco smoke condensate studies.

1003545151

8. Cont. from page 2.

This extensive modification of fine structure of F1 histone is also observed in regenerating liver. (Balhorn et al, 1971) Considerable microheterogeneity due to phosphorylation of F1 is seen in rapidly dividing Morris rat hepatomas, mouse mammary carcinoma, Lewis mouse lung tumor, mouse skin melanoma, and Shear mouse glioma in log phase culture growth versus stationary phase. (Balhorn et al, 1972b). There is also a linear correlation between growth rate and F1 phosphorylation in the Morris series of minimal deviation hepatomas.

Others have shown (Gurley et al, 1973) that massive F1 and F3 phosphorylation occurs during the G2-M transition in cultured Chinese hamster ovary cells.

Langan has demonstrated a more limited and specific phosphorylation of F1 which is stimulated in liver by glucagon or cyclic AMP administration (Langan, 1968). Two amino acid sites - ser 37 and ser 106 - have been identified as the phosphorylated residues, but only the ser 37 site is modified by a cAMP- (and presumably glucagon-) dependent enzyme. This group has also demonstrated a 40-100% increase in template activity in vitro by reconstituting chromatin with this specifically phosphorylated F1 histone. (Watson and Langan, 1973) Others have shown an increase in ³H-Actinomycin D binding, as well as increased template activity, in calf thymus chromatin preparations treated with pineal gland protein kinase (Fontana and Lovenberg, 1973).

Because of the foregoing evidence, it seems probable that histones, especially lysine-rich F1, play a structural role in the maintenance and regulation of chromatin and that hormonally-mediated or proliferation-associated phosphorylation allows access of polymerizing enzymes to naked DNA by 1) removal of the proteins or 2) relaxation of steric or configurational restraints on the chromatin (possibly-supercoiling). This latter suggestion is supported by work (Adler et al, 1971) showing that in reconstituted F1-DNA complexes, phosphorylated F1 is less effective than the unmodified species in altering the DNA contribution to the circular dichroic spectrum of the complex, i.e. there is less interaction between phospho-F1 and DNA.

Evidence is also accumulating that levels and patterns of phosphorylation of other, acidic nuclear proteins (non-histone chromosomal proteins, NHCPs) are directly related to various parameters of differentiation or gene activation. Several groups (Shea and Kleinsmith, 1973; Kostraba and Wang, 1972; Chae et al, 1972; Martelo et al, 1970) have demonstrated tissue-specific patterns of NHCP phosphorylation as well as tissue-specific spectra of NHCPs. Allfrey (1973) proposes a role for NHCP phosphorylation in the regulation of transcription and cites as evidence several key observations: 1) phosphorylated NHCPs

1003545152

stimulate transcription in vitro from DNA of homologous tissue, 2) dephosphorylation destroys this stimulatory effect, 3) uptake of ^{32}P in vivo correlates with gene activation in PHA-stimulated lymphocytes and ecdysone-treated insect chromosomes, 4) in liver, cAMP and cortisol stimulate a selective phosphorylation of specific NHCPs, 5) cells with low levels of RNA synthesis, e.g. mature avian erythrocytes, display decreased levels of NHCP phosphorylation, and 6) in synchronized HeLa cell cultures, the lowest levels of NHCP phosphorylation correspond to the G2-M phase of the cell cycle when RNA synthesis is at a minimum.

Evidence for phosphorylation-mediated activation of a specific protein of known function is provided by the work of Martelo (1973) who reports that rabbit skeletal muscle protein kinase stimulates DNA-dependent RNA polymerase from *E. coli* (with T4 phage DNA as template) by selectively placing a phosphate on a single serine residue of the sigma factor.

Taken together, all of these observations strongly suggest that aberrations in the control of nuclear protein phosphorylation may be crucial in the etiology of neoplastic growth. Histone phosphorylation, with its correlation to cell division rate, and NHCP modification, with its relation to gene activation, are thus important to a complete understanding of the control of cell function.

As in the past, a clear definition of the nature of metabolic processes has depended on characterization of the associated enzymatic activities. Therefore, we propose to initially carry out an investigation of the phosphorylating enzyme(s) (protein kinases or PKs) which catalyze these protein modifications in mammalian tissues. So far PKs which phosphorylate histone have been found in a wide variety of species and tissues. (Kuo and Greengard, 1969). cAMP-dependent kinases have been implicated in control of mammalian protein synthesis, (Walton et al, 1971) bacterial RNA synthesis, (Eron et al, 1971) and mammalian synaptic transmission, (Greengard, 1973) as well as in glycolysis (activation of phosphorylase b). As of this writing, the enzymes responsible for NHCP and histone phosphorylation are poorly characterized, although the following mechanism is generally accepted (Kumon et al, 1972; Gill and Garren 1971; Corbin et al, 1972) $\text{RC} + \text{cAMP} \rightleftharpoons \text{R-cAMP} + \text{C}$, where R represents one or more regulatory subunits, C is the active catalytic subunit, and RC represents the inactive holoenzyme.

Several groups have reported the isolation of PKs from the nuclear compartment of various cell types; this finding is important in light of the importance of phosphorylated macromolecules in the nucleus. Reddon and Anderson (1972) have isolated four cAMP-

1003545153

independent PK activities from rat liver by phosphocellulose chromatography. Our laboratory has also resolved four enzymes, using DEAE chromatography (see below under Preliminary Work). It seems highly likely that there should be at least two distinct kinase activities in nuclei, since enzymes have been partially purified which prefer either histones (Lake and Salzman, 1972; Takeda et al., 1971; Siebert et al., 1971) or NHCPs (Martelo, 1973) but not both. The conflicting reports in the literature concerning cAMP-dependence of kinases isolated by various laboratories undoubtedly stems in part from transient or cell compartmental fluctuations in cAMP levels (i.e., adenylate cyclase or cyclic nucleotide phosphodiesterase activities) or artifactual concentration changes introduced because of dilution during preparation. These dilutions could affect the equilibrium reaction described above. Other enzymes whose presence may hamper interpretation of data are the ATPases and phosphatases which are present in most subcellular fractions, including nuclei. (A discussion of these problems and how they can be overcome is given in Corbin et al., 1973a,b)

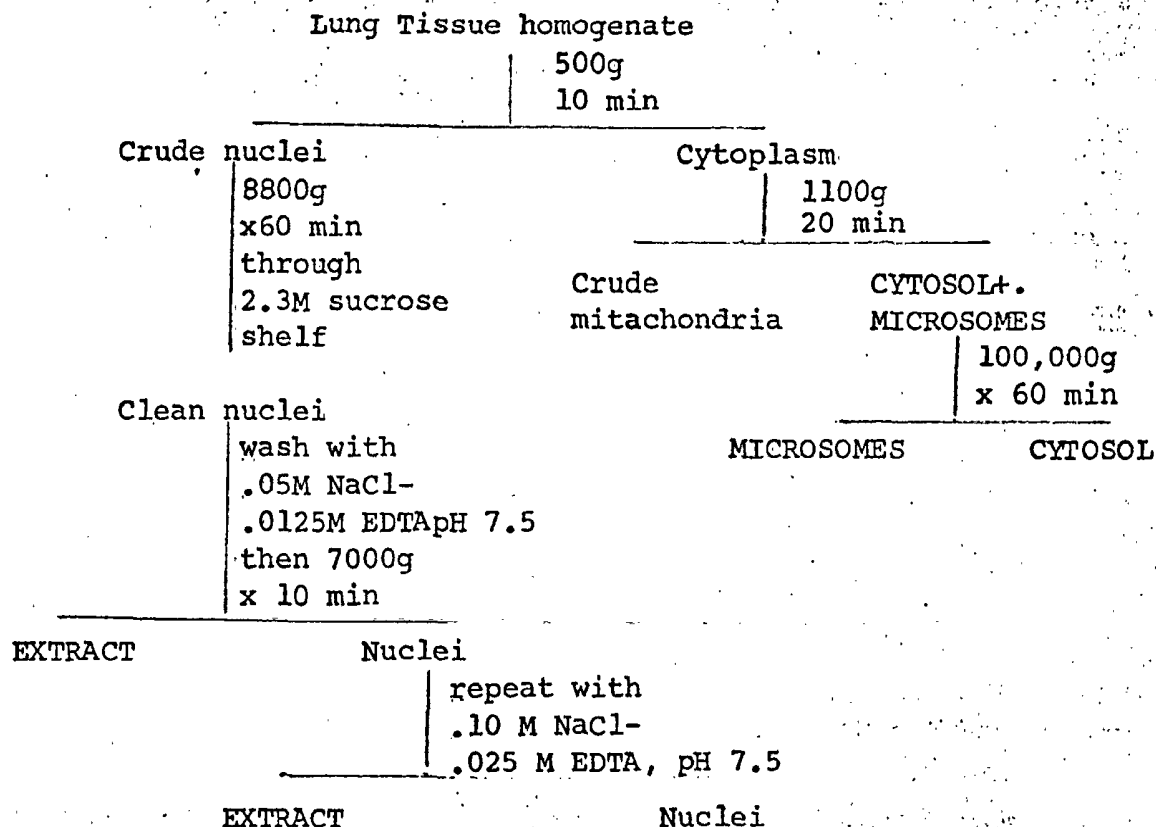
The system we have chosen for study is the C57BL/6 mouse, an animal we have studied for several years for possible age-dependent changes in chromatin template activity (O'Meara and Herrmann, (1972)). Early experiments will require only normal animals; later experiments will require carcinogen-treated and tumor bearing animals. At that time we plan to treat a portion of our mouse colony with benzo(a)pyrene, tobacco smoke condensates, and urethane and to examine isolated chromatin proteins and protein kinases.

Because our mouse colony is presently being maintained as an aging colony, we plan also to examine the effect of age of both carcinogen-treated and untreated animals upon the levels of nuclear protein phosphorylation and protein kinase activity in liver and lung tissues.

Preliminary experiments have been carried out with rat liver with the goal of developing a satisfactory method of extracting protein kinase activity from whole nuclei, prior to an investigation of kinetics, substrate specificity, and regulation. Nuclei prepared by the Blobel and Potter method from rat liver have been carried through the successive Tris washes described by Huang and Huang 1969 for solubilization of chromatin. This technique involves two rinses in .075 M NaCl-.025 M EDTA, pH 8, followed by two washes in each of the following Tris buffer solutions, also pH 8: .05 M, .01 M, .002 M, .0004 M, and finally distilled water. Each wash begins by dispersion of the preceding pellet with a Potter-Elvehjem homogenizer and ends with a 7000g centrifugation. When these extracts were assayed for kinase activity, it was found that 62% of the total nuclear PK activity was solubilized by the first three washes (two saline-EDTA and one .05 M Tris wash). This activity was stimulated three-fold by 5×10^{-6} M cAMP. The surprising finding that so much activity

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could be released by such mild treatment of nuclei prompted us to repeat this type of experiment with dog lung tissue which possesses a higher degree of connective tissue infiltration, to insure that the technique would serve for the proposed lung studies. Sub-cellular fractions were prepared according to the following scheme:



The nuclear extracts were pooled and the subcellular fractions designated by UPPER CASE letters were assayed for protein kinase activity in our standard assay (see Methods section for details). The data shown in Table I verifies that low salt washing is sufficient to extract considerable protein kinase activity from whole lung nuclei. Also shown is the fact that the protein solubilized is much richer in PK activity than any cytoplasmic fractions. Experiments with these pooled nuclear extracts have allowed us to determine approximate pH optimum (6.0) (see figure 1) and K_m for cAMP ($5 \times 10^{-7}M$) (see figure 2). These values doubtless represent several histone-phosphorylating enzymes in dog lung cell nuclei and they will be re-evaluated as we fractionate and purify the enzymes. A similar study was also carried out with rabbit kidney cells to examine the effect of infection with herpes virus HSV-1. A significant difference between infected and non-infected cells was observed, as indicated in Figure 3.

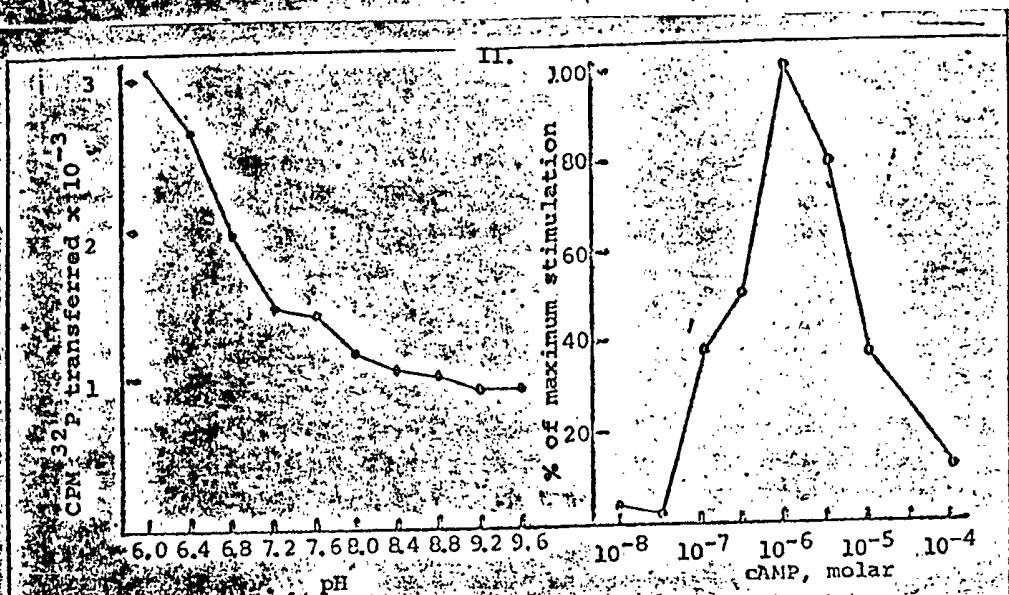
1003545155

In an attempt to further resolve nuclear PK activities, we have chromatographed the .05 M and .10 M NaCl extracts this time from nuclei of rat liver, on DEAE-cellulose. Figure 4 below shows the apparent resolution of four PK activities clearly. It can be seen that the proteins extracted by .10 M NaCl are qualitatively similar to those in the .05 saline extract (the UV-absorbing material eluted at high ionic strength - 0.5 M KCl - probably represents nucleic acid precursors and low molecular weight RNA). Also to be noted is the marked enrichment of the PK (peak III in figure 3) eluting at 0.3 M KCl. This peak III is currently under investigation.

Table I. Protein kinase activity in subcellular fractions of dog lung.

Enzyme source + or - 5 uM cAMP	µg protein	pmoles ³² P transferred	pmoles ³² P µg protein	cAMP stimulation
Combined nuclear extracts (+)	9.2	25,900	2815	1.6-fold
Combined nuclear extracts (-)	9.2	16,100	1750	
Cytosol plus microsomes (+)	395	99,400	252	3.7-fold
Cytosol plus microsomes (-)	395	27,000	68	
Cytosol (+)	375	277,000	741	4.0-fold
Cytosol (-)	375	68,900	184	
Microsomes (+)	120	4,900	41	2.2-fold
Microsomes (-)	120	2,300	19	

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Figures 1 and 2. CAMP concentration and pH dependence of protein kinase activity in pooled .05 M and .10 M NaCl nuclear extracts from dog lung (standard assay as described in Methods). In fig.1 CAMP = 5×10^{-6} M; in fig.2 pH = 6.5.

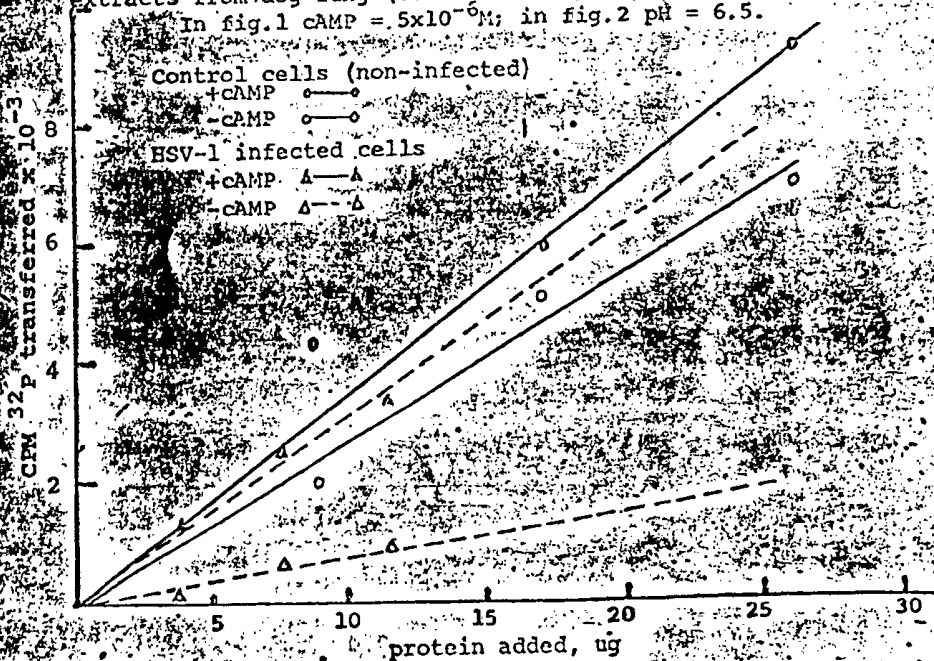


Figure 3. Log-phase rabbit kidney cells were harvested, sonicated, and assayed for PK activity in standard assay. Graph shows linear dependence of activity on added cell protein. When included, CAMP was 5×10^{-6} M.

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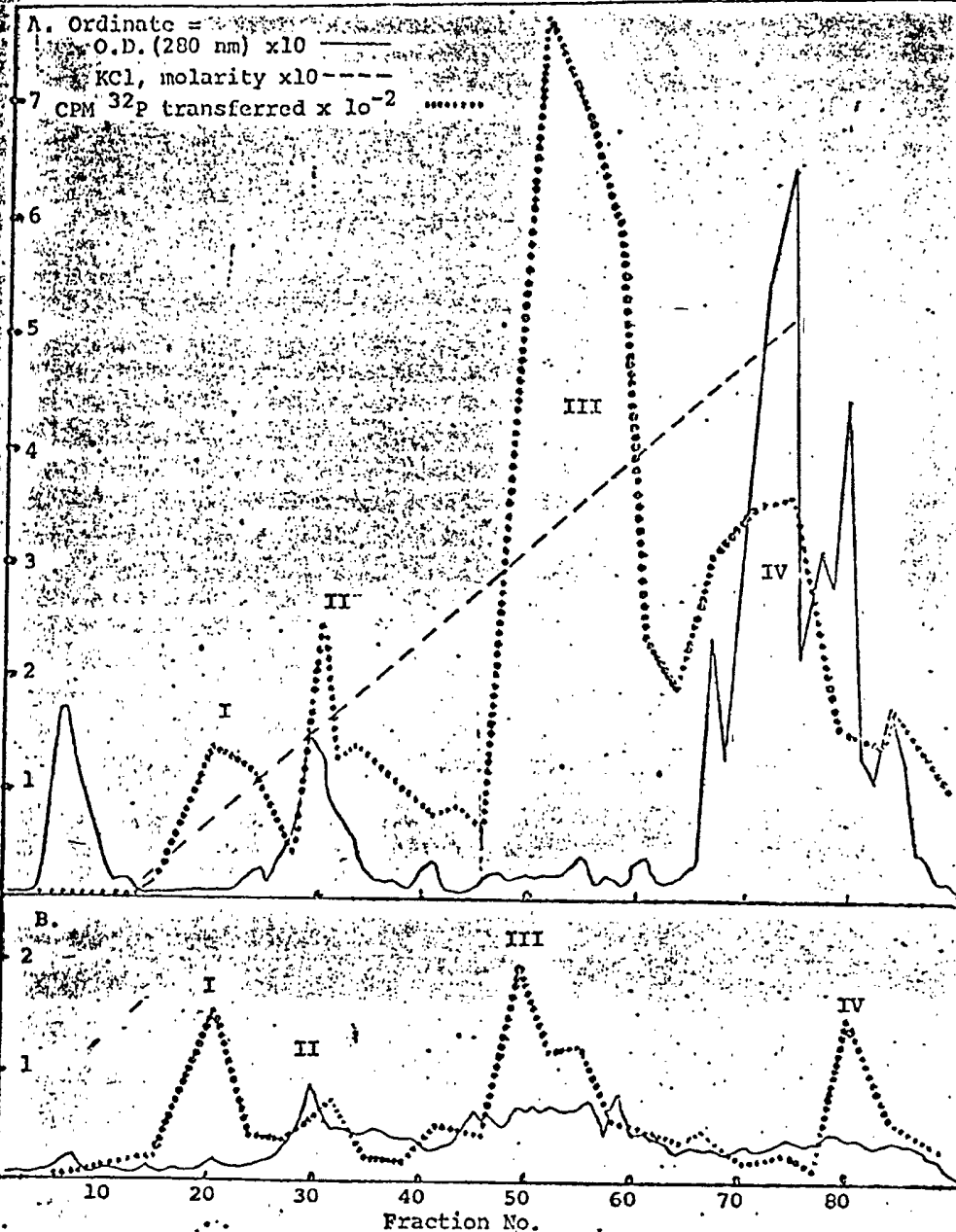


Figure 4. Approximately 3 mg of .05 M (Panel A) and .10 M (Panel B) NaCl nuclear extracts was applied to a 1×10 cm DEAE-cellulose column and eluted with a 100 ml gradient of 5-500 mM KCl, 10mM Tris, pH 7.4. Fractions were assayed for protein kinase activity (standard assay described in Methods) with cAMP included at 5×10^{-6} M.

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2. Experimental Procedures

a) Preparation of Tissues

C57BL/6 mice will be obtained from Jackson Laboratories, Bar Harbor, Maine. Animals will be maintained in the animal laboratory of the V.A. Hospital, Bedford, Mass. All other operations will be carried out at the Boston University Medical Center.

b) Nucleoprotein preparation and analysis

Cell Nuclei will be prepared in this laboratory by the method of Blobel and Potter (1966). Tissues are dissected as free as possible of connective tissue and, in the case of tumors, necrotic patches. This is followed by extensive washing in isotonic sucrose (0.25M). The tissue is finely minced, homogenized in a Dounce glass homogenizer with loose pestle, and filtered through cheesecloth. Crude nuclei and cytoplasm are prepared by a 10-minute, 500xg centrifugation. Clean nuclei are prepared by centrifugation of the crude nuclear pellet (after dispersion with the loose pestle in 1.7M sucrose) through a shelf of 2.3M sucrose. Nuclei prepared in this manner are relatively free of membranous cytoplasmic contaminants as verified by phase contrast microscopy. This method has proved quite adequate for our work with rodent tissues (rat and mouse) and for our preliminary studies with dog lung. We are currently exploring an alternate nuclear preparation method based on the work of Magliozzi et al. (1971), who have overcome the difficulty of working with mammalian tumor tissue by the inclusion of low concentrations of lead (.002M PbAc₂) and Triton N-101, a non-ionic detergent, in initial fractionation steps. It is claimed from electron microscopic evidence that this method yields absolutely clean nuclei while inhibiting nuclear autolysis and enzymatic degradation of chromosomal proteins (with a yield of 90%). Naturally, the effect of Pb⁺⁺ on PK activity will be determined. Another alternative is the Chauveau method (1956) as it has been modified for tumor tissue by Siebert (1967) or Busch et al (1968).

Whole histones will be isolated from nuclei by the acid extraction technique of Panyim et al (1971). F1 histone will be extracted from nuclei, whole histones or chromatin by the Johns method (1964). The lysine-rich F1 fraction will be further purified and fractionated on Bio-Rex 70 columns eluted with a stepwise gradient of 8-40% guanadinium HCl. (Hohmann and Cole, 1971) Electrophoretic analysis of histones will be carried out on pH 2.3 acrylamide-urea acetic acid gels. Purified lysine-rich

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histones are resolved into F1 phosphorylated derivatives by 40hr. electrophoresis on 25cm gels. (Panyim and Chalkley, 1969) When desired, F1 histones are dephosphorylated by treatment with E. coli alkaline phosphatase. (Balhorn et al, 1971). β -mercaptoethanol and NaHSO_3 will be included in the histone preparative buffers to inhibit aggregation (primarily formation of F3 dimers) and histone protease activity, respectively.

A mild treatment for the isolation of NHCP's will be employed, involving extraction of purified nuclei with 1.0 M NaCl followed by DEAE-cellulose chromatography. (Wang, 1967; Kamiyama and Wang, 1971)

Since this method resolves significant quantities of phosphoproteins, alkali-labile phosphate content will be determined (Kleinsmith et al, 1966; Berenblum, 1938). NHCPs will be fractionated on SDS-Tris gels according to the method of Van den Broek et al (1973). There is a gentle method for isolating histones and NHCPs from the same tissue, which we are currently experimenting with. Chromatin is prepared from nuclei by successively decreasing ionic strength Tris buffer washes until the chromatin is finally solubilized in distilled water (Huang and Huang, 1969) It is then sheared in a Virtis homogenizer in 3.0M NaCl; protein is separated from DNA on a Bio-gel A-50m column. Histones are removed from the pooled protein peaks (after concentration) by chromatography on Bio-Res 70 and the NHCPs comprising the run-off from this column are fractionated on DEAE-cellulose. (Elgin and Bonner, 1972). All gels will be analyzed after staining for determination of protein bands using a Gilford spectrophotometer equipped with a gel-scanning attachment.

c) Protein kinase isolation, assay, and characterization

Our standard assay for protein kinase activity is a modification of the method of Greengard et al (1969). It contains, in a total volume of 0.2 ml : 15 mM potassium phosphate buffer, pH 6.5; 2.5mM NaF; 0.3 mM EGTA; 2.0 mM theophylline; 6.2×10^{-8} M ATP having 10^5 - 10^6 dpm of AT^{32}P ; 500 μg of calf thymus whole histone; and enzyme preparation. When called for, cAMP is added at 5×10^{-6} M. Incubation time, except for time-course studies, is fifteen minutes at 37°C . The reaction is stopped by addition of 2 ml of ice cold 20% TCA-0.25% NaWO_4 containing 0.1 M Na_3PO_4 . 0.63% bovine serum albumin is added (0.2 ml) as a carrier protein. The precipitate is removed by means of a ten minute, 2500xg centrifugation and resolubilized in 1.0M NaOH. This procedure is repeated twice and the final precipitate is counted in NaOH and Aquasol in a liquid scintillation spectrometer. For determination of enzymatic incorporation

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of phosphate into endogenous protein, the histone addition is omitted from the above procedure.

Desired, F1 histones are dephosphorylated by treatment with D. Col. We have employed several methods of PK preparation. Total soluble PK can be prepared by the Greengard method. Nuclear PK activity has been prepared by a variety of techniques. (Takada et al, 1971; Lake and Salzman, 1972; Siebert et al, 1971) All involve salt extraction of nuclei. A further purification step involves chromatography on a phosphocellulose or DEAE cellulose column. We find that extraction of nuclei twice with .075M NaCl-.025 M EDTA, pH 8, followed by two washes with 0.05 Tris pH 8 is sufficient to extract more than 60% of the kinase activity from preparations of rat liver nuclei. We plan to determine optimum salt concentration, pH of extraction, and cellulose chromatography conditions for the isolation of this soluble or weakly bound fraction of kinase activity, as well as the residual PK activity, which is either chromatin- or nuclear membrane-bound. We will determine the feasibility of an early gel filtration or DEAE-Sephadex step to separate holoenzyme from activated catalytic subunit and cAMP bound regulatory subunit. We are also investigating the possibility of preparing an affinity column of purified F1 histone coupled to Sepharose 4B. (Cuatrecasas and Anfinsen, 1971) Enzyme preparations which have been purified through one or two DEAE steps will be passed through this affinity column and PK will be specifically eluted with excess F1 histone. Alternative purification procedures will include isoelectric focussing or sucrose gradient centrifugation. All enzyme preparations will be assayed for ATPase and phosphodiesterase activity by the method of Corbin et al (1972). Since pre-existing levels of activation prior to PK preparation are important in dictating the physical state of the holoenzyme (and since degree of activation of cAMP dependent PK is related to cAMP levels) all tissues will be assayed for cAMP with the assistance of Dr. Peter Polgar, of our Department of Microbiology, who has extensive experience in the measurement of cyclic nucleotide concentration and adenylyl cyclase activity. (Polgar et al, 1973). Whole tissues, of course, will also be assayed for gross levels of PK activity and cyclic nucleotide response.

Purified PK activities will be electrophoresed (on SDS gels), centrifuged in sucrose gradients, and run on Sephadex columns to determine molecular weights. Kinetics of ^{32}P transfer will be examined and optimum temperature determined. Optimum metal ion concentrations and ionic strength will be investigated as well as the K_m for cAMP, ATP, and substrate.

In order to determine natural substrates for the kinase activities isolated, we will add whole histone or NHCP prepared

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from mouse tissues to the standard protein kinase assay in lieu of calf thymus histone. The final TCA precipitate will then be divided and electrophoresed on identical urea (for histones) or SDS-Tris (for NHCPs) gels. One gel will be stained and scanned for protein. The other will be sliced and counted for determination of the extent of ^{32}P incorporation into protein peaks. In addition, we will attempt to utilize the new method of two-dimensional gel electrophoresis (Orrick et al, 1973) of nuclear proteins to determine differences between control cells and cells from carcinogen-treated animals. This technique, which yields better resolution of NHCPs than ordinary one-dimensional electrophoresis, should allow us to determine cancer-specific phosphorylated nuclear acidic proteins. On the other hand, our current gel system for histones is quite adequate for investigation of these proteins. Alternatively, nucleoprotein fractionated by the methods described in the preceding section will be utilized as substrate in the standard assay. Protein will be determined in the TCA precipitate, normalized with respect to assay conditions and protein concentration, and in this manner relative substrate preference of the enzyme(s) will be ascertained.

d) Carcinogen Studies

Following the isolation and partial purification of the nuclear PK's we next propose to examine the effects of various carcinogens which have been implicated in the alteration of the structure and function of DNA and to compare their effects with those of tobacco smoke condensates. These known carcinogens include alkylating agents such as the nitrosamides and alkane sulfonates which have been shown to act directly at the DNA and RNA levels to bring about alkylation of the N7 position of guanine, the N1 and N3 position of adenine and the N1 position of cytosine. In addition, we will examine the ethylating reagent nitrosoethylurea which has also been shown to directly alkylate the N7 position of guanine. These studies have been reviewed by Magee and Barnes (1967). Inhibition studies will be carried out with levels of carcinogen which approximate the physiological concentrations effective in carcinogenesis. Comparison with tobacco smoke condensate will be carried out using the preparative technique of Sydnor, Allen and Higgins (1972).

Although the nitrosamides and alkane sulfonates are believed, generally, to function directly, without the requirement of metabolic conversion to an active form, it is planned to examine their effects in the intact mouse with the expectation that there may well be significant effects which are secondary to their alkylating activities

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which may depend upon additional metabolic steps. Mice will be injected with N-Methyl-nitrosourea (≈ 100 mg/kg body weight) or methylmethane sulfonate (50 mg/kg body weight) and liver and lung tissue removed and treated for the isolation of nuclear phosphoproteins and PK's as described above. We will attempt to

Because the carcinogens of the N-nitrosamine group have been detected in tobacco smoke condensate (Rhoades and Johnson, 1972), we also plan to study the very potent carcinogen, dimethylnitrosamine (20mg/kg body weight) which is known to require prior metabolic conversion to an active form. Here, BALB/c mice will be used as well as C57BL/6 mice, and newborn mice will be used as well as adult animals (Toth et al, 1964). Comparison with the effects of whole tobacco smoke condensate will be carried out with the water-soluble extract prepared as described by Sydnor et al (1972), administered in the dose range 0.25-1.0 mg/ml of 5% sucrose solution in the drinking water. Special care will be devoted to the possible presence of new or altered PK's in the carcinogen-treated animals; any such enzymes would be thoroughly examined as a possible target for a new chemotherapeutic approach.

which appear to be at physiological concentrations in rodents and

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e. Potential Importance of this Work to the Cancer Problem

Injected with N-Methyl-nitrosourea (MNU) The etiology of cancer has been variously associated with cell alterations leading to cell proliferation or dedifferentiation. We have attempted to show the crucial position which nuclear protein phosphorylation appears to occupy in the regulation of these metabolic processes. Our proposal involves the characterization of the enzymes and substrates involved in the phosphorylation process, and includes a comparison both in vitro and in vivo of carcinogenic agents, which are known to function at the nuclear level, with tobacco smoke condensate. Our overall goal is to seek for new or altered nuclear phosphorylating enzymes which appear as the result of carcinogen treatment, to fully characterize these activities and to describe the mechanism of their production.

f. Previous work by applicant

Past studies from this laboratory which relate to this proposal follow two lines. We have been interested in the effects of the acridine dyes upon the replication of the episomal F factor of E. coli strain K-12. (Samaha et al 1967) and our most recent results have posed the interesting possibility that the acridines can function as inhibitors of DNA polymerase I in E. coli not only by binding to the DNA substrate but also by binding - apparently at the active site - to the enzyme. (Eberhard and Herrmann, 1972). Our second line of interest has covered possible age-dependent modifications in the structure and template activity of mouse liver chromatin (O'Meara and Herrmann, 1972). We therefore feel confident that we can carry out the enzyme isolation and kinetic studies outlined in this proposal, and we have had considerable experience with the preparation of mouse chromatin and its fractionation into histone and non-histone components.

We have also worked with the cyclic AMP-dependent differentiation of the mouse C1300 Neuroblastoma cell in tissue culture (Burstein, Neumann and Herrmann 1972) and this has given us some appreciation for the intricacies of cyclic AMP involvement in metabolic control processes.

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- c. Potential Importance of this Work to the Cancer Program
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9. The proposed work would be carried out in the laboratory of the principal investigator in the Medical Research Building of the Boston University Medical Center. The laboratory occupies 1200 sq. ft. and includes a room for high voltage and disc gel electrophoresis and a large laboratory for enzymology and protein fractionation. There are also adjacent facilities shared with two other investigators; a large cold room, a hot room, and a preparation room. Equipment in the laboratory of the principal investigator includes a liquid scintillation spectrometer, Gilford spectrophotometer with linear transport for gel scanning, fraction collectors and an RC-2B centrifuge. Also available are a Technicon amino acid analyzer, Spinco L2-50 and Model E analytical ultracentrifuges, and an RCA-EMU-3G electron microscope.

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24.

11. Biographical Information: Principal Investigator

Robert L. Herrmann, Ph.D.

Associate Professor of Biochemistry

Boston University School of Medicine

Boston, Massachusetts 02118

Academic Preparation:

B. S. Purdue University

Ph.D. Michigan State University

post-Doctoral M.I.T.

Honors:

Damon Runyon Postdoctoral Fellow, M.I.T. 1956-1958

REDACTED

Professional and Scientific Organizations:

REDACTED

Research and/or Professional Experience:

1965-present: Associate Professor Biochemistry

Boston University School of Medicine

1964-present: Consultant in Biochemistry, V.A.

Hospital, Bedford, Mass.

1959-1965: Assistant Professor of Biochemistry,

Boston University School of Medicine, Boston.

1958-1959: Research Associate, M.I.T., Boston.

1956-1958: Damon Runyon Postdoctoral Fellow, M.I.T.

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Five Most Relevant Publications:

1. The Effect of Iododeoxyuridine upon Conjugation and the Fate of Transferred DNA in *E. coli* K-12. A.D. Cooper, M.W. Burgan, C.W. White and R.L. Herrmann, *J. Bacteriol.*, 107 433 (1971).
2. Effect of DNA Ligands on Deoxyribonucleases and DNA Polymerase I of *E. coli* K-12. C. Eberhard and R.L. Herrmann *J. Bacteriol.* 112 224-230 (1972).
3. Cyclic AMP-Mediated Increase in Acetylcholinesterase Activity in Neuroblastoma cells in vitro. S.A. Burstein, J.R. Neumann and R.L. Herrmann, *Federation Proc.* 31 3561 Abs. (1972).
4. A Modified Mouse Liver Chromatin Preparation Displaying Age-related Differences in Salt Dissociation and Template Activity. A.R. O'Meara and R.L. Herrmann, *Biochem. Biophys. Acta*, 269 419-27 (1972).
5. Effect of Iododeoxyuridine on Cell Division in Recombination-deficient Mutants of *E. coli* K-12. D.M. Hanson & C.W. White and R.L. Herrmann. Abstracts, 73rd Ann. Mtg. Amer. Soc. Microbiol. Miami Beach 1973.

1974-present Consultant in Biochemistry. V.A.
Hospital, Bedford, Mass.

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#956-KLEINERMAN

1003545172

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

February 11, 1974

Grant application No. 956

CANCER

To: The committee comprising Drs. Gardner, Huebner, Meier

Subject: Jerome Kleinerman, M.D., St. Luke's Hospital, Cleveland
New application No. 956
"Studies of Carcinogenesis in Organ Culture of Trachea
and Bronchi"

History

Dr. Kleinerman's current CTR grant, No. 857R1, runs to June 30, 1974 and is in a terminal year. It is on a different topic, experimental emphysema.

Request

Application No. 956 requests \$36,904, plus one additional year.

Document Submitted

Attached is application dated Feb. 4, 1974 (32 pages).

Comment

Dr. Kleinerman's bibliography suggests that cancer is a new interest of his.

FWN:wg
Encl.

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F.W.N.

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THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

Date: Feb. 4, 1974

1. Principal Investigator (give title and degrees):

Jerome Kleinerman, M. D.

2. Institution & address:

Saint Lukes Hospital, 11311 Shaker Blvd., Cleveland, Ohio 44104

3. Department(s) where research will be done or collaboration provided:

Pathology Research

4. Short title of study:

Studies of Carcinogenesis in Organ Culture of Trachea and Bronchi

5. Proposed starting date: 7-1-74

6. Estimated time to complete: two years

7. Brief description of specific research aims: The specific overall objectives of the work proposed in this research application are to develop an optimal system of organ culture of hamster trachea, bronchi and bronchioles and of human trachea, bronchi and bronchioles which will permit survival of the cells and tissues in as near an optimal state as possible (compared to precultured tissue) for a period of time of sufficient duration to develop malignant neoplasms following the introduction of single pure chemical carcinogens within the culture medium; to evaluate methods of determining viability within the explants of trachea and bronchi; and to determine the true malignant character of any tumor which may be induced in the in vitro system by re-inoculation or transplantation into a living animal, and observation and study of the implant for growth and metastases. Studies in isolated systems, such as those proposed, allow for more specific and direct evaluation of the true time course, the target cell of origin, and the opportunity for biochemical evaluation of the earliest lesion associated with premalignant and overtly malignant tissue. Ultra-structural studies may also provide an indication of the cell type, the organelles affected and the temporal course of the effects of several carcinogens on the cell organelles.

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8. Brief statement of working hypothesis:

2.

The purpose of the present research proposal is to develop an optimal system of organ culture for maintenance of trachea, bronchi and bronchioles (lung tissue) for prolonged periods and to develop methods of inducing carcinogenesis in the cultured trachea and bronchiolar tissue by exposure to carcinogenic chemicals in culture. This work is based upon the premise that respiratory tissue in organ culture will respond more specifically to stimuli induced by carcinogenic chemicals toward the development of malignant transformation. Relatively isolated systems eliminate hormonal influences, immunological effects and other systemic factors including perhaps viral infections which can influence malignant transformation in vivo. Attempts will be made to produce a system in which the tissue explants will survive for longer periods and may therefore have greater opportunity to develop malignant transformation under the influence of chemical carcinogenic stimuli. Both human and animal models will be studied in order to compare their effects and transfer observations when they are useful. Realistic and critical means of evaluation of suspected neoplastic transformations will be utilized to evaluate this change. Confirmation of the neoplastic transformation will include histological, ultrastructural, biochemical and transplantability criteria. If these objectives are accomplished, a standardized and reliable method of observing the early cellular and organoid changes in the development of a malignant neoplasm will be provided, and a manipulable and reproducible model for inducing and studying the initial and progressive alterations in the afflicted cell or cells will be available for both biochemical and morphologic (including ultrastructural) study. A by-product of this research will be the development of standardized and uniform methods for testing the effect of various chemicals for carcinogenic potential on respiratory tract epithelium.

9. Details of experimental design and procedures (append extra pages as necessary):
A. Background:

The technique of organ culture offers a unique compromise in the study of chemical carcinogenesis. It eliminates the many confusing limitations in working with whole animals and yet preserves the organoid community of cells and tissues which together give any organ its unique structural and functional identity. Organ culture of lung was first practiced by Fell (1) on embryonic organ fragments and femurs. In 1959 Trowell (2) systematically evaluated the maintenance and growth of mature organs in an artificial culture system. Trowell demonstrated that epithelial cells did better when massed with minimal stroma and that mitoses could be observed in epithelium. He specifically sustained lung and noted, as was our experience, that the alveolar tumors are overgrown by enlarged alveolar epithelial cells. Lasnitzki (3) studied the effect of 3-4 benzpyrene on human fetal lung in organ culture and showed the outgrowth of bronchioles, alveoli and cartilage. The carcinogen produced bronchiolar hyperplasia and abnormal mitoses but inhibition of connective tissue growth. Similar changes were observed by this same author using four fractions of cigarette smoke condensates, but different types of epithelial cell hyperplasia and metaplasia were observed with the various types of condensates. Cailleau, Crocker and Wood (4) reported the long term culture of human bronchial mucosa and neoplasm in 1959. Ciliary activity was observed for as long as 135 days and mucous material in glands up to 21 days. Tumor tissue did not survive for long periods. Sorokin (5) described the development in organ culture of mammalian fetal lung. Most components of lung developed but vascular tree growth was restricted. He noted nearly full potential for lung formation and differentiation. The effects of "epithelial growth factor" (EGF) on organs in organ culture were described by Jones (6). His studies suggested stimulation of lung growth by EGF, but careful control studies were not performed. Simnett and Heppleston (7) reported significant differences in mitotic index of lung alveolar tissue in organ culture relating to sex, strain and age differences. After three days of culture, these differences were reduced; these authors suggested that sex and age cultures may be hormonally induced but strain differences were persistent and inherent. These same authors (8) studied the mitotic incidence (MI) in lung alveolar tissue of newborn, three and twelve month mice in organ culture for 22 days. They concluded that cell division of lung alveolar tissue is controlled in part by a tissue inherent mitotic inhibitor, present in adult lung but lacking in neonatal, in association with other stimulation factors possibly hormonal. Sorokin and Adelstein (9) reported the effect of 1100 rads of x-ray given 24 hours before explantation

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on the development of organ cultures of lung. Ciliogenesis appeared to proceed normally from centrioles (redimentary cilia) and basal bodies still unformed after X irradiation, even though the cell proper has suffered a dose which will impair its proliferation. Davis (10) described a somewhat different technique for the maintenance of guinea pig lung in organ culture. His method consisted of a flask-like apparatus with two stopcocks. Within the chamber the tissue is supported, nourished and oxygenated. By this method the lung was maintained for 14 days; after 6-7 days the alveolar spaces became solidly filled with alveolar epithelial cells. Blood vessels (except for endothelium) and bronchioles are well maintained. Ultrastructural study confirmed the normalcy of the cellular structures. Lasnitski (11) observed that a hydrocarbon rich fraction from cigarette smoke condensate uniformly caused the growth of new bronchi, caused epithelial enlargement, stimulated mitoses, and induced bronchial epithelial hyperplasia in human fetal lung in organ culture. The effect was more widespread in younger tissue. Dirksen and Crocker (12) reported the ultrastructural alterations of respiratory epithelium produced by polycyclic aromatic hydrocarbons on suckling rat trachea in organ culture. Several potent carcinogens including 7, 12, dimethylbenz(a)-anthracene (DMBA) and benzo(a)-pyrene (BP) produced cells with little endoplasmic reticulum, many free ribosomes, abundant cytoplasmic filaments and autophagic vacuoles. DMBA produced these abnormalities at low concentrations in contrast to the other compounds. Cherry and Taylor Robinson (13) reported a method for the production of large quantities of tracheal organ cultures in roller type tubes. This has not been attempted for large scale production of lung explants. These studies indicate the complete feasibility of successful organ culture of lung tissue.

The subject of bronchopulmonary carcinogenesis has been reviewed recently and some of the facts relevant to this investigation will be considered in the following discussion (14,15,16). There is a considerable lag in progress made on cancer of the respiratory tract when compared with other organs and systems. The difficulty in experimentally inducing respiratory tumors has been overcome lately with the introduction of the Syrian golden hamster as the animal of choice by Della Porta, Kolb and Shubik (17). These authors produced bronchogenic carcinoma in hamsters by repeated intratracheal applications of a suspension of dimethylbenzanthracene in a 1% gelatin colloid. These observations were later extended to benzpyrene in Tween 60 or olive oil (18). These results could not always be reproduced and therefore an important step ahead was taken when Saffiotti, et al (19), injected intratracheally into hamsters a suspension of a mixture of benzpyrene and hematite. This method consistently produces a high number of respiratory tract tumors in vivo, with generally negligible inflammation or irritation. For successive papers, this group of investigators has carefully studied the conditions of their model (20,21). It has become evident by then that in the lung, as in skin and other organs, the vehicle or solvent used was of paramount importance in the induction of tumors. Kuschner (22), for example, has shown that large, repeated intratracheal doses of benzpyrene without carrier dust failed to produce cancer in hamsters while 3-methylcholanthrene given in the same manner was a potent respiratory carcinogen. To date no systemic study on the role of vehicles in the production of bronchogenic tumors has been reported. Such studies are included in the present proposal. As a result of their use of wire-mesh pellets impregnated with polycyclic hydrocarbon carcinogens and studies on the particle size of the carcinogen and its carrier dust, Kuschner (23) and Saffiotti (24) respectively have considered the possibility that the carcinogenetic potency of a given compound may be related, among other factors, to its retention by the bronchial tissues. Experiments in this proposal are designed to obtain information that, hopefully, will clarify the matter.

Saffiotti's group has recently demonstrated that pharmacological doses of vitamin A can inhibit considerably the induction of tumors by benzopyrene - Fe₂O₃. A similar

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observation had been made in 1943 by Rosicky and Hatschek on mouse-skin tumorigenesis by benzpyrene. The biochemical mechanism of this phenomenon is difficult to evaluate; several possibilities for further studies are outlined here.

In 1958, Magee and Barnes (25) discovered the carcinogenicity of dimethylnitrosamine. N-nitroso compounds not only occur in foods and tobacco smoke, but can be found in vivo. Tracheobronchial tumors have been produced in hamsters by the administration of diethylnitrosamine by lavage, feeding or intratracheal injection (26). Dontenwill and his colleagues (27) have extensively studied this alkylating agent and have shown that it is a systemic carcinogen with positive organotropism for the liver in rats and for the respiratory tract in the hamster, regardless of the route of administration.

In vitro studies on respiratory tract carcinogenesis have met with only limited success. As Kuschner and Laskin (23) point out, "...even here convincing changes beyond the induction of striking atypical epithelial alterations have not been achieved." A vast literature is available on mechanisms of carcinogenesis in other organs (liver, skin, hematopoietic system), much of which is probably applicable to the respiratory system (see for example, references 28,29,30). However, because of its peculiar morphology, function and biochemistry, such applicability will have to be established.

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B. Choice of Animal Species for Study

1. Organ culture techniques will be explored to evaluate the best combination of external gaseous environment, media, temperature or incubation and method of culture. The tissue of choice will be that of the Syrian hamster. This animal has been widely accepted as the one of choice for in vivo studies involving experimental production of lung tumors for several reasons. First, the incidence of spontaneous development of pulmonary neoplasms is exceedingly low, and second, this animal has great natural resistance to infection and is not easily subject to incidental pulmonary infections. Moreover, because of the ultimate necessity for re-transplantation of any tumors generated in organ culture into intact animals of the same species, animals of an inbred strain should be utilized. One such strain exists in this country. This strain Bio #8720, is obtainable from the Trenton Experimental Laboratory Animal Company, Bar Harbor, Maine. Study of organ cultures of trachea and lung tissue from suckling and adult (80-100 gm) hamsters of this inbred strain will be utilized. In addition to studying the trachea and bronchus, the peripheral lung tissue will be cultured. The purpose of the lung tissue culture is to evaluate the survival and integrity of the intrapulmonary bronchioles. In our experience these structures are well preserved in organ cultures and have all the advantages of remaining within their natural pulmonary environment within a lung slice, while containing all of the epithelial structures characteristic of bronchial or bronchiolar structures. For these reasons they represent an excellent source of bronchiolar tissue for study.

2. Human Bronchial and Bronchiolar Tissue

Human material from surgical and autopsy cases where spontaneous disease is minimal or absent will also be used in the organ culture studies. Because of the close proximity and intrinsic relationship of the autopsy and surgical pathology service to the investigator, material from selected cases may be available for culture within 20-30 minutes after death. This in large measure is due to the well organized pathology assistant staff which is an integral part of the autopsy room staff at St. Luke's Hospital and to the immediate supervision and control of autopsy material by the investigator. Other studies involving pediatric and neonatal tracheal material have been successfully used by collaborating investigators at affiliated institutions for organ culture studies. This attests to the acceptability of the human material and the ease in obtaining it immediately after death.

C. Method of Performing Organ Culture Studies

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1. The primary method of organ culture to be explored will be that of Trowell. In this method, to be described in greater detail in the body

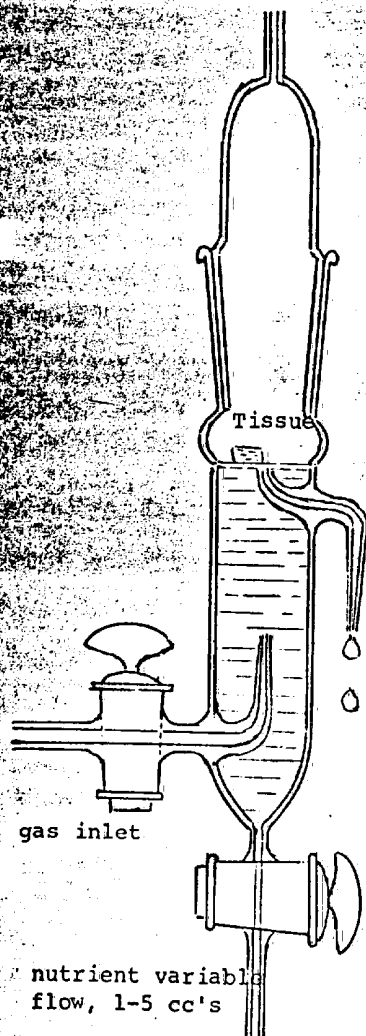
of this section, the tissue fragment or explant is supported on lens paper or rayon on a metal grid, the bottom surface of which is immersed in culture fluid.

2. Modifications of the Trowell (2) technique will also be studied. These will include the attachment of the tissue fragment to the base of a plastic petri dish which is scarified with a knife blade. Multiple fragments are placed in various separate locales on the Petri dish surface and submerged in culture fluid.

3. The Cherry Technique (13). In this method tracheal, bronchial or lung fragments are placed in roller tubes in 1-1.5ml. of culture fluid and rotated continuously. This technique will be evaluated for preservation of the tissue and maintenance of good cell integrity.

4. The Modified Davis Technique (10).

In this method, reported by Davis in 1967, the lung tracheal or bronchial tissue is maintained on a platform of rayon or wire mesh which is supported (like a fritted glass filter) on the bottom half of a glass chamber. This glass chamber is connected by two stopcocks to the nutrient medium source and to the atmospheric gas. The top portion of the chamber leads via a millipore filter to the outside atmosphere. This technique is worthy of further modification and study because it is small and readily adaptable to a slow, continuous perfusion system for nutrient and atmospheric gas supply to the fragment of tissue. This methodology may be useful in attempting to maintain viability, of the organ culture fragment for periods of over 21-28 days. It would appear to this investigator that alteration of the Davis technique to provide continuous pump-fed nutrient supply at a very slow rate, and a continuous flow system for atmospheric gas are two of the vital parameters which must be explored in attempts to prolong the viability of the cultures.



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5. In addition to the above methods, the following variation in culture parameters will be attempted to produce optimal tissue survival conditions.

a. Evaluation of the Gas Tensions in the Atmosphere.

While many studies of organ culture of lung and other tissues have utilized atmospheres of 95-97% oxygen and 5-3% CO₂, recent studies have suggested that this concentration of oxygen may be injurious even to respiratory tract epithelium for long periods. In studies soon to be reported (Boat, Kleinerman, et al) (31) the principal investigator has had the opportunity to evaluate the effects of 95-100% oxygen on human tracheal tissue in organ culture. Prolonged exposure to this high oxygen atmosphere produces unequivocal injury to mucosal epithelium and glands. Preliminary studies suggest that it would be more reasonable to keep the atmospheric oxygen concentration at 50-70% O₂ and 50-30% nitrogen and 3% CO₂. (Note: 40-50% would probably be optimal in vivo, but the increased concentrations will be helpful in providing adequate oxygen tensions in the lower layers of the submerged cultures.)

b. Evaluation of the Effects of Temperature.

Recent studies have suggested that cultures of trachea and bronchi may be better maintained at 32-34°C. instead of the usual 37°C. This temperature will be evaluated.

c. Variations in culture media.

In recent years investigators have attempted to simplify the complex supplemental medias used originally in organ culture. In the past we have successfully cultured hamster lung and trachea using Trowell's T-8 media supplemented with chick embryo extract, horse serum and antibiotics. Since a sizable quantity of cultured tissue will be necessary for adequate viability and biochemical assays, it would seem economically desirable to utilize a simplified growth media. Therefore, we propose to evaluate the following medias using the Trowell, modified Trowell, roller tube and modified Davis chamber methods of culture:

1. Trowell T-8, horse serum, penicillin and streptomycin with and without 10% or less chick embryo extract, pH 7.0-7.2;
2. 199, horse serum, penicillin and streptomycin with and without 2% or less chick embryo extract, pH 7.0-7.2;
3. Basal Eagles Medium (buffered with Hepes), penicillin and streptomycin with and without 0.2% or less bovine albumin, pH 7.0-7.2. The use of the Hepes buffer will maintain the original pH of the media for up to 15 days using the roller tube technique for tracheal cultures, as suggested by Cherry and Taylor-Robinson. (13).

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d. The Effect of Vitamin A

The role of vitamin A in maintaining the integrity of mucosal and epithelial surfaces has been classically demonstrated by the work of Fell and Mellanby (32), Laznitski (33) and Aydelotte (34). Laznitski has demonstrated the effectiveness of vitamin A in maintaining mucosal characteristics in in vitro situations. More recently Saffiotti (19) and associates have reported the inhibitory effect of vitamin A on the induction of squamous metaplasia and neoplasms in hamsters by combinations of benzpyrene and hematite administered intratracheally. The concentrations of vitamin A in the culture media will be varied in order to evaluate those doses which are optimal for preservation of epithelial integrity. Vitamin A will be utilized in final concentrations of 10-20 international units per milliliter (0.01-0.02 millimoles). Media deficient in vitamin A will contain a total absence of this material. Vitamin A will be introduced into the culture medium in the form of vitamin A alcohol (13-Cis-retinol). The systematic investigation of 30-50 I.U./ml of culture media will also be studied. This data will be useful in evaluating the effects of higher doses of vitamin A when they are used in conjunction with carcinogens introduced directly into the culture media.

6. Monitoring of media for bacterial and mycoplasma infection.

Growth media will be cultured at the time of harvest of the explant for bacterial or mycoplasma infection. Any contaminated organ culture will be immediately eliminated from further analysis.

D. New Methods to be Investigated in Conjunction of Organ Culture Approaches

1. Separation and cultivation of mucosa after detachment from cartilage and adventitial tissue.

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Full thickness sections of human trachea may at times exceed 2 mm of thickness. This excessive accumulation of tissue elements may interfere with the proper nutrition of the submucosal elements, both epithelial and connective tissue. Better nutrition will be achieved when cartilagenous tissue and excessive adventitial connective tissue can be removed. Techniques to effect this separation have been successfully applied in our laboratory. The mucosa, superficial connective tissue, and glands can generally be separated from underlying cartilage. We have accomplished this in human tracheal tissue and animal tissue other than hamster where an adequate amount of submucosal tissue and glands exist.

Technique: A #30 stainless steel needle is introduced between the submucosal and cartilage and a balanced salt solution is gently injected to create a plane of cleavage. Gentle dissection is used to separate the mucosa and glands from the underlying cartilage, which will then be cultured separately by the organ culture techniques previously described.

2. Effect of macrophages with and without hematite and benzapyrene on the maintenance growth of tracheal and bronchial organ cultures.

The recent observations of Saffiotti, Cefis and Kolb (19) have suggested that the interaction of dust particles and macrophages may influence the localization of neoplasmas in the bronchial tree generated by the introduction of dust and benzapyrene into the tracheobronchial tree. It has been suggested that the carcinogen absorbed on the dust particle surface penetrates the respiratory epithelium and is phagocytized by macrophages. It is eluted from the macrophages by plasma and macrophage disintegration and diffused along the mucosal surface to impregnate the deeper mucosal layers at various sites in the bronchial tree. This hypothesis has not completely evaluated the role of the macrophage in the processing of the carcinogen into an active state nor its role in delivering carcinogens to the lower layers of the epithelium by process of active motility and insinuation within the cells of the mucosa. These possibilities can be evaluated using an organ culture system with trachea. The general outline of the study will be as follows:

Macrophages will be harvested from the lung of each animal whose lung will be subsequently cultured. If this technique appears to injure the lining of the epithelium and interferes with the proper growth of the trachea in culture the technique will be modified to harvest macrophages from the peripheral bronchi and lung without exposing the major portion of the trachea to the washout process. Autologous macrophages harvested by perfusion of lung with normal saline will then be centrifuged and resuspended in growth media with and without serum. Aliquots of macrophages will be incubated with the following materials: 1) hematite dust of 99% less than 10 micra in diameter, 94.1% less than 1 micra in various concentrations so as to produce optimal macrophage ingestion of the particulate, 2) mixture of equal weights of benzapyrene (purified) and hematite ground together in a mortar to yield a finely divided homogeneous dust containing 50% each of benzapyrene and hematite by weight, 3) a solution of benzapyrene alone dissolved in acetone and diluted with growth media to final concentration equal to that present in the particulate form, 4) macrophages exposed to an inert dust such as carbon black in sufficient concentration to produce ingestion in considerable quantities by the macrophages, and 5) macrophages alone without dust or benzapyrene exposure.

Autologous tracheal explants (the same animal from which macrophages were collected) will be exposed to concentrations of 1×10^6 macrophages containing various combinations of hematite, carbon and benzpyrene and hematite, benzpyrene and macrophages alone. The effects of single exposures will be evaluated over the life period of the organ culture. Since limited numbers of explants are available, harvest will only be performed in the preliminary studies at weekly intervals.

In another series of experiments again utilizing autologous macrophage and tracheal explants the longitudinal effects of dust alone in macrophages, benzpyrene plus dust and benzpyrene alone are studied by

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utilizing harvesting at 2,4,7,14,21 and 28 days and at weekly intervals thereafter. One hamster will be used for the longitudinal evaluation of each of the variables to be studied. This will provide an adequate number of explants to allow for harvest and study of explants over longer periods of time. At least five or six hamsters will be used to evaluate each of the variables.

The ultimate goal of this study will be to analyze the role of the alveolar macrophage in the processing of carcinogen benzpyrene and its ultimate effect in inducing carcinogenesis. The role of the so-called inert dust, hematite, may also be evaluated as compared to the carbon black, since epidemiological studies reported from Great Britain in hematite miners suggest an increased prevalence of primary lung cancer in this group.

E. Morphological Evaluation

1. Periodic harvest and histological evaluation.

At predetermined intervals organ culture explants of trachea, bronchi and lung will be harvested, fixed in neutral buffered formalin or Bouin's solution and processed by conventional techniques of 4-6 micra thickness. Harvest interval in its early experimental studies will be 1,2,4,7,14,21, and 28 days and at weekly intervals thereafter for as long as cultures are viable or cultures are available for harvest. Our present technique allows for at best twelve segments of hamster trachea in the form of half rings to be available from each animal. These will be distributed among different culture dishes so that each dish will contain an equal number of fragments from upper, middle and lower portions of the trachea. This sampling will be utilized to avoid any local effects related to trauma or differences in trachea metabolism. A similar number of explants from peripheral lung tissue which includes medium sized bronchi and bronchioles will be cultured and harvested simultaneously. In addition to regular histology, special stains to evaluate the acid mucopolysaccharide (AMP) of the cultured tracheal cells will be performed. Alcian blue/PAS at pH 2.5 and pH 1.0 with and without sialidase digestion. In addition, Baker stain for bound phospholipids will be performed to evaluate the secreting abilities of the Clara cell in the bronchioles. The general character of the epithelium, the height and size of the cells, the relative proportion of each cell type, the mucus secreting abilities of the secretory cells and the cytologic character of each of the cell types will be evaluated by light microscopy techniques. In addition the naphthylamidase (35) reaction will be performed on selected explants which will be harvested from each of the various experimental studies. The naphthylamidase technique is performed on frozen sections at pH 5.5 to demonstrate lysosomal cathepsin B activity. This histochemical method is said to demonstrate cell injury and lysosomal enlargement in a stage much before changes can be demonstrated by other techniques. By means of this technique sections

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incubated with Leu-2-naphthylamide (LNA) at pH 5.5 in a 0.1 molar acetate buffer for 1-2 hours in the primary reaction produce 2-naphthylamide, simultaneously coupled to diazo blue B and heated finally with CuSO_4 to produce a blue or violet chelated end produce. This test can provide evidence of early cell injury and act as an index of incipient cell damage.

2. 1 micron Epon embedded section

When it has been established that reasonable cell survival and integrity has been accomplished in the culture system by light microscopic analysis, additional studies utilizing 1 micron thick Epon embedded sections and light microscopic analysis will be performed. By means of this technique a relatively rapid but more detailed analysis of cell types and cell characteristics will be observed. In this analysis morphometric techniques for evaluating the relative proportion of cell types, cell size and intracellular organelles will be evaluated.

3. Ultra thin sections for electron microscopy

When necessary ultra thin sections for electron microscopy will be prepared from the tissue embedded in epon for more detailed analysis of cell membrane, ciliary characteristics, mitochondrial structure, number, size and character of liposomes, amount and distribution of rough and smooth endoplasmic reticulum and the number and size of characteristic secretory granules. This analysis will be performed on both control and carcinogen exposed cultures as a means of evaluating the optimal organ culture system to support the long growth of tracheal, bronchial and lung explants.

4. Autoradiography

Cell viability and replication will be evaluated by the application of radioautographic methods.

1. Tritiated thymidine incorporation (H^3 Thymidine). The uptake of this isotope will be used as an index of DNA synthesis. Incorporation into the nuclei of viable replicating cells occurs during the "S" phase of the cell cycle. Tritiated thymidine will be introduced into the culture medium in concentrations of 0.05 $\mu\text{C}/\text{ml}$ or 0.10 $\mu\text{C}/\text{ml}$ (specific activity 1.9 C/mmole). This low concentration is used so as to produce no radiation injury to the cells. After a pulse label of 2-4 hours, the labelled thymidine will be removed and fresh, nonlabelled culture medium replaced. No chaser of cold thymidine will be utilized since this technique may tend to arrest further progress towards division of mammalian cells in S phase or G_1 -S transition. At intervals following the pulse label, explants will be harvested, fixed and prepared for histologic sectioning.

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These sections will be dipped in NTB-2 emulsion in the dark and placed in light tight boxes for 3-4 week exposures. After development they will be evaluated for thymidine index and/or labelled mitoses. For these studies it does not appear necessary to determine the true "S" time or cell cycle period, since our interest is in determining the state of viability, rather than the exact cell cycle parameters. However, the autoradiographs of the sections will be evaluated for nuclear labelling in each of the specific cell types, including: ciliated cells, mucous secretory cells, goblet cells, basal cells, intermediate cells and Clara cells when they are present.

Pulse labelling will be performed at various times during the cultivation of the tracheal or lung (bronchiole) fragments. Periods for study may include 18-24 hours, 40-48 hours, 90-96 hours, 1 week and at weekly intervals until culture is discontinued. This technique will also be utilized by pulse label technique after exposure to various carcinogens and at the specified intervals during culture indicated for the control or noncarcinogen cultures.

5. Biochemical evaluation of viability of organ cultures

Synthesis of proteins and lipids and protein secretion are acceptable indicators of energy requiring reactions in the living tissue. For determinations of protein synthesis, the fragments of trachea and lung will be incubated with uniformly labeled L-leucine- C^{14} . Incubations will be performed for 90-120 minutes at $37^{\circ}C$ in rubber stoppered flasks containing 95% oxygen and 5% carbon dioxide in the gas phase. The reactions will be stopped by chilling, and after removal of the medium and repeated washing of the tissues, trichloroacetic acid (to a final concentration of 10%) will be added. After homogenization and centrifugation, the sediment will be washed with 5% cold trichloroacetic acid. Removal of lipids, phospholipids and RNA will be done according to Massaro, Weiss and Simon (36). The resulting protein precipitates will be dissolved in 1M sodium hydroxide; an aliquot will be used for protein determination by the method of Lowry et al (37) while another aliquot will be assayed for acid insoluble radioactivity. Results will be expressed in dpm/mg protein. Alternatively, incorporation of S^{35} methionine in the epithelium of trachea and lung fragments, can be examined (38).

Lipid synthesis is another parameter than can be utilized in the assessment of the viability of certain tissues. Although no information is available concerning trachea, Nasr and Heinemann (39) have demonstrated that mammalian lung tissue can incorporate acetate, palmitate and glucose into various lipids, in vitro. Fragments of lung and/or trachea will be incubated with uniformly labeled acetate C^{14} or palmitate C^{14} for two hours at $37^{\circ}C$. At this time HCL will be added to a final concentration of 0.02N and the flasks shaken for 20 minutes. The tissue will be washed, homogenized and extracted by the method of Folch, Lees and Stanley (40). An aliquot will be taken for analysis of total lipid

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radioactivity in a liquid scintillation counter. Results will be reported as micromoles of substrate per gram wet weight per hour.

The secretory activity of trachea and lung fragments can be measured by the isolation of isotopically labeled protein-polysaccharide complexes, such as "complex A" of Kent et al (41). Fragments of trachea or lung will be incubated at 37°C for 2-4 hours in medium containing D-glucosamine-C¹⁴ or sulphate -S³⁵, which are precursors of the complex. At the end of the incubation period the spent medium will be removed and applied on a Sephadex G-200 column. Aliquots will be collected for scintillation counting. Because of its high molecular weight, radioactivity incorporated into the complex is recovered in the void volume while the remaining radioactive molecules are eluted later. Results will be expressed in micromoles of substrate per gram wet weight per hour.

A normal baseline and range will be initially established for all these biochemical parameters of cell function by first examining their values in fresh tissues, immediately after death and before culture. These will be compared with those obtained at different times during organ cultures in the presence or absence of carcinogens in the various vehicles tested. The effect of vitamin A will be also studied.

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6. Radioactive Isotopes for Metabolic Incorporation

Other isotopic tracers may be utilized to indicate cell viability by incorporation into metabolic products. For these studies sulfur 35 as an inorganic sodium sulfate will be utilized, and incorporation into macromolecules of acid mucopolysaccharides will be observed. Because of the energy in the sulfur isotope Beta emitter, distinct localization by audioradiography is not possible. However, similar studies utilizing tritium or carbon labeled glucosamine or L fucose can be used to study incorporation by audioradiographic techniques into the intracellular macromolecular polysaccharides. Similarly, tritiated palmitate can be used to evaluate the incorporation of phospholipid precursors into the intracellular structures of the lung. This precursor is particularly useful in evaluating synthesis of phospholipid dipalmitoyl lecithin which is believed to be synthesized in the laminated bodies of the Type II cells. Finally, the incorporation of radiolabeled carcinogenic materials will be utilized to study the incorporation, transportation and localization of these materials within the mucosal epithelial cells by audioradiography.

7. Morphometric analysis

In order to evaluate in a quantitative fashion the results of prolonged culture and effects of carcinogens, a culture tissue morphometric technique will be applied. These techniques will permit more detailed quantitative evaluation of the following parameters:

- a) Number and distribution of cell types;
- b) Size, including height and area of specific cell types;
- c) Nuclear size and nuclear cytoplasmic ratio of specific cell types, i.e., mucous secreting cells, basal cells;
- d) Number and size of mitochondria per unit of cell area;
- e) Number and area of lysosomal structures per unit cell area in each specific cell type;
- f) The amount of smooth and rough endoplasmic reticulum and free ribosomes per unit cell area in each specific cell type; and
- g) The number, size distribution, and area of secretory granules present per unit area of secretory cell at different times during the course of the culture.

All of these analyses can be performed on the specific cell types at different times during the course of culture, and similarly at varying times after exposure to carcinogens. Morphometric analysis will be performed utilizing photographic enlargement of one micron thick Epon embedded sections of tracheal explants or electron micrographs of these structures. The techniques are those of Weibel and associates (42,43). Additional morphometric studies will be performed by image analysis methods by the application of the Quantimet 720B. This sophisticated instrument will allow us to study parameters relating to area, intercept and count from grey level analysis of histological preparations, utilizing either one micron sections or regular paraffin sections. This apparatus will provide a semi-automated method for performing these quantitating but exceedingly time consuming analyses.

8. Use of carcinogens

A variety of carcinogens have been utilized both in vitro and in vivo

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In respiratory tract epithelial tissues to induce preneoplastic and neoplastic changes. In vivo studies utilizing dimethyl 1-2 benzantracene (DMBA) in colloidal suspension (17), 3,4 benzpyrene in an aqueous Tween 60 or in olive oil (18) or sesame oil, 3-methylcholanthrene (22) have been observed to produce neoplasms by direct instillation into the tracheobronchial tree, while systemic administration of diethylnitrosamine or dimethylnitrosamine are observed to produce large numbers of epithelial tumors in the upper respiratory tract, the stomach and the larynx. Saffiotti and his associates have recently produced lower respiratory tract neoplasms by injections of 3-4 benzpyrene and hematite suspensions. Herrold (26) and others have reported the development of upper respiratory tract neoplasms after the administration of other n-nitroso compounds. In vitro studies utilizing polynuclear hydrocarbons have been reported by Lasnitzki (11), utilizing 3-4 benzpyrene by Palekar, Kushner and Laskin (44), utilizing 3-methylcholanthrene by Dirksen, and Crocker (45) utilizing 7-12 dimethylbenz(a)anthracene (DMBA), benzpyrene, methylcholanthrene and other less carcinogenic materials. To date these in vitro studies have produced abnormal states of epithelial differentiation or increased proportions of activity dividing basal cells. The ultrastructural alterations produced by DMBA and other carcinogens have been reported by Dirksen and Crocker (45). These changes consist of cytoplasm with little endoplasmic reticulum, many free ribosomes, complex autophagic vacuoles and abundant cytoplasmic filaments.

Therefore, it seems reasonable to utilize three of these carcinogenic hydrocarbons as prototypes for study of their effects on the respiratory mucosa in organ culture. These will be, a) 3,4 benzpyrene, b) 3-methylcholanthrene, c) diethylnitrosamine (DEM), and d) a relative noncarcinogen, 1,2,3,4 dibenzanthracene. 3,4 benzpyrene will be added according to the technique of Crocker (45) as an acetone solution to the growth media in a final concentration of 0.4% acetone. The actual concentrations will range from 10-15 micrograms per milliliter. Benzpyrene can also be utilized in a solution of Tween 60. The explants will be exposed continuously to this material both with and without supplementation by Vitamin A, using doses previously described.

3-methylcholanthrene will be prepared according to the techniques of Palekar, Kushner, and Laskin (44) in a solution of Tween 60 containing 2.3 to 5.0 micrograms of methylcholanthrene per 100 milliliters of media. 1,2,3,4 dibenzanthracene (DMBA) will also be prepared according to the technique of Crocker (45) in an aqueous solution of similar concentrations containing 2.3 micrograms of DMBA per 100 milliliters of media and containing 0.1 milliliter of Tween 60.

Diethylnitrosamine (DEM) will be utilized in an aqueous solution according to the technique of Herrold (26), Montesano and Saffiotti (46) in a concentration of 1.0 to 5.0 mg per 100 ml of tissue culture media. This dose will be adjusted on the basis of the observed effects in organ culture.

These carcinogenic agents will be studied one at a time by the methods indicated. Evaluation of their effects will be performed by periodic harvest and histologic methods previously outlined. If continuous exposure to these substances produces irreversible injury, lower doses will be evaluated. In studying the effects of 3,4 benzpyrene, the effects of this material in acetone solution and in a suspension both with and without hematite and macrophages will be evaluated according to the protocol.

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9. Criteria for the evaluation of malignant transformations in organ culture

Criteria for the evaluation of malignancy will be dependent on three points:

a. Histological effects of organ culture. Evidence of malignant

transformation will be evaluated on the evidence of dysplastic and truly neoplastic changes within the epithelial structures. Criteria will be epithelial metaplasia and dysplasia, loss of differentiation of the epithelial cells, pleomorphism and anaplasia of the epithelium, invasion of neoplastic cells into gland ducts and the adjacent subcutaneous tissue and exfoliation of dysplastic and neoplastic cells into the culture fluid. Epithelial change suggesting "an intraepithelial carcinoma" will also be acceptable as preliminary evidence of a neoplastic change. These changes include a more uniform but less differentiated cell type which replaces the entire epithelial surface and may extend into the gland ducts.

b) The transplantability of organ culture explants will demonstrate neoplastic characteristics into inbred hamsters of the same strain. Explants which appear to have neoplastic changes will be minced into fragments no larger than 1 mm cubed and introduced subcutaneously or intramuscularly into both adult and suckling hamsters. The injected animals will be observed for growth of tumor locally and possible metastatic spread.

c) If it should grow, the transplanted tumor will be re-evaluated by histological techniques to make certain that it is the epithelial and not the mesenchymal elements which are proliferating. If growth after transplantation does not occur, the recipient animals can be prepared by cortisone pretreatment or X-irradiation prior to transplantation in an attempt to minimize any immunologic mechanism which could be invoked against the transplanted tumor. This will be done in spite of the fact that the immune processes are not likely to be stimulated toward rejection in this inbred strain. Finally, transplantation will be attempted in an internal organ, such as the lung, which is said to be a more protected environment for growth of transplanted neoplasma than peripheral sites. If, on retransplantation, all attempts to stimulate growth are ineffective, we will attempt to dissociate the neoplastic cells and grow them in isolated culture free from mesenchymal elements. If this culture technique is successful, the epithelial component will be harvested in sufficient quantities for transplantation attempts into subcutaneous tissue and lung.

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THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

February 11, 1974

Grant application No. 980

CANCER

To: The committee comprising Drs. Gardner, Huebner, Meier

Subject: Michael Ming-Chiao Lai, M.D., Ph.D., University of
Southern California, Los Angeles
New application No. 980
"Interaction of Chemical Carcinogens and Avian RNA
Tumor Viruses"

History

A preliminary inquiry was handled as Case No. 236 and full application was encouraged.

Request

Application No: 980 requests \$29,923, plus two additional years.

Documents Submitted

Attached is application dated 1/21/73 (sic), received February 1, 1974.

FWN:wg
Encl.


F.W.N.

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THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

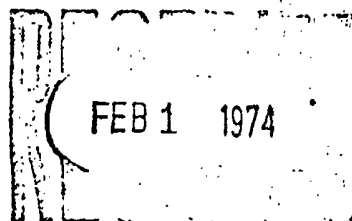
110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8985

Application for Research Grant
(Use extra pages as needed)

Date: 1/21/73

1. Principal Investigator (give title and degrees):

Michael Ming-Chiao Lai, M.D., Ph.D.
Assistant Professor of Microbiology



2. Institution & address:

University of Southern California
University Park
Los Angeles, California 90024

3. Department(s) where research will be done or collaboration provided:

Department of Microbiology
University of Southern California School of Medicine
2025 Zonal Avenue
Los Angeles, California 90033

4. Short title of study:

Interaction of chemical carcinogens and avian RNA tumor viruses

5. Proposed starting date: July 1, 1974

6. Estimated time to complete: June 30, 1976

7. Brief description of specific research aims:

1. To determine whether a chemical carcinogen can activate sarcoma genes in cells by identifying and characterizing virus particles released from methylcholanthrene-transformed quail tumor cell lines.
2. To determine whether sarcoma virus could derive sarcoma-specific genes ("oncogenes") from cells by studying changes of the virus genome when leukemia virus is serially passaged in methylcholanthrene-transformed cells.
 - (a) Polyacrylamide gel electrophoresis will be used to test whether the leukemia viral RNA increases in size.
 - (b) By oligonucleotide fingerprinting technique, sarcoma-specific gene sequences acquired during the course of passage in chemically transformed cells will be looked for.
 - (c) Leukemia virus passaged in chemically transformed cells will be tested for focus forming activity.
3. To determine whether the transformation-specific glycoproteins and glycoproteins of avian sarcoma virus are coded for by the sarcoma virus or result from the transformed state of the cells, by comparing glycoproteins and glycopeptides of sarcoma and leukemia viruses released from chemically transformed and control cells.

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3. Brief statement of working hypothesis: 2. There is some circumstantial evidence suggesting that chemical carcinogen, such as hydrocarbon compounds, and RNA tumor virus can act synergistically to cause cellular transformation. In some *in vitro* studies, the exposure of tissue culture cells to both the hydrocarbon carcinogen and murine leukemia virus transformed the cells while the exposure to either one alone did not (1, 2). The most likely explanation is that the chemical carcinogens alter or activate some cellular genes, or so-called "oncogenes" (3), which then act in concert with the viral genes in the leukemia virus. We have no knowledge as to how the cellular oncogene and viral genes interact.

It has been postulated that RNA tumor viruses can transduce cellular information, for instance, oncogenic genetic material (4). The phylogenetic histories of several strains of murine sarcoma viruses suggest that this is a real possibility: Kirsten sarcoma virus was isolated after prolonged passage of Kirsten murine erythroblastosis virus in rats (5), and Moloney sarcoma virus was isolated by passage of Moloney murine leukemia virus in mice (6). Furthermore, it has been shown that Kirsten sarcoma virus contained some transformation-specific genes of the rat cell lines (7). Thus it is likely that the sarcoma viruses arise by recombination between cellular transformation genes and the leukemia viral genome. However, no experimental system has been established which allows for the direct monitoring of this hypothetical recombination between virus and cell.

I propose to study the interaction between cellular transformation genes and RNA viral genomes by studying the evolution of leukemia virus during serial passage in methylcholanthrene-transformed cells. It is believed that sarcoma virus or the virus with sarcomagenic characteristic might be recovered from such cultures. The rationale behind this approach is that the chemical carcinogens might activate certain cellular transforming genes which can then be incorporated into superinfecting leukemia virus. I would concentrate on studying focus-forming activity and the structure of the viral RNA and envelope of the virus released from chemically transformed cells, since the RNA and envelope glycoproteins of sarcoma virus have been found to be distinct from those of leukemia virus (8, 9, 10).

Details of experimental design and procedures (append extra pages as necessary) The basic design of this project is to infect methylcholanthrene-transformed quail cell lines with several strains of avian leukemia viruses. The viruses released from such cell lines will be studied with regard to their infectivity, transforming capability, and their structure, especially their RNA and glycoproteins. Serial passages of leukemia viruses in these cell lines will be followed. Changes in the viral characteristics monitored, particularly oncogenic properties, will be indicative of an effect of chemically transformed quail cells on the virus. These changes will be carefully investigated to reveal a possible causal relationship with cell transformation by chemical carcinogens. Parallel passages in normal quail fibroblasts will serve as controls.

BACKGROUND

A. Differences between avian sarcoma viruses and avian leukemia viruses.

Avian RNA tumor viruses can be roughly divided into sarcoma viruses and leukemia viruses according to their pathogenicity. Generally, sarcoma viruses transform chicken embryo fibroblasts when grown in tissue culture, while leukemia viruses do not (8). Since the transformed cells cause neoplasms upon injection into susceptible animals and also possess several other properties characteristic of tumor cells *in vivo* (11), sarcoma viruses are generally regarded as possessing oncogenic capability and sarcoma virus-transformed cells are used as a model for studying oncogenesis *in vitro*.

The isolation from avian sarcoma viruses of conditional mutants which affect only their transforming ability (12, 13, 14, 15, 16) suggests that the avian sarcoma viruses carry genes which are responsible for neoplastic transformation. The absence of such sarcoma-inducing genes from avian leukemia viruses is reflected not only in their inability to transform chicken embryo fibroblasts *in vitro* but also in two structural aspects of viruses: RNA and glycoproteins.

a. RNA: The virion RNA of avian RNA tumor viruses contains 1 molecule of 70S single stranded RNA plus several molecules of 4-10S RNA. The 60-70S RNA is considered to be the viral genome and has a complex structure (17). It consists of probably three to four pieces of 30-40S subunits (18, 19) and some low molecular weight RNA (20, 21, 22). The latter may represent cellular RNA but may also include specific linkers for the 30-40S pieces as well as a primer RNA for reverse transcription (20, 23). The 30-40S RNA of avian tumor viruses can be resolved electrophoretically into 2 classes, a larger one a and a smaller one b (8). Cloned nondefective avian sarcoma viruses contain only class a RNA; whereas leukemia viruses consist solely of class b RNA (24, 10). Transformation defective viruses derived from avian sarcoma viruses and biologically identical to leukemia

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viruses also contain only class b RNA (8). It was therefore suggested that class a RNA carried genetic information which is specific for the formation of sarcomas in the animal and for foci in fibroblast culture (8).

Furthermore, I have shown with Sanger's two-dimensional fingerprinting (electrophoresis at pH 3.5 and homochromatography) that all sequences demonstrable in class b RNA are also present in class a RNA. In addition, class a RNA appears to contain sarcoma-specific sequences which are not present in class b (10). Thus, sarcoma virus RNA can be distinguished from leukosis virus RNA by the following two ways:

- (1) Electrophoresis in polyacrylamide gels reveals that sarcoma virus RNA moves slower than leukosis virus RNA.
- (2) Two-dimensional fingerprinting reveals that sarcoma virus RNA contains sarcoma specific sequences which are not present in leukosis virus RNA.

These two methods will be used in this project to detect any change in genetic information in the genomes of avian leukosis viruses and to detect viruses with sarcomagenic capability.

b. Envelope glycoproteins: The avian RNA tumor virus contains an envelope which determines the host range, interference pattern and type-specific antigenicity of the virus (25). The viral envelope is composed of lipids which are mostly derived from the host membranes (26), and glycoproteins, which are coded for by the viruses (27, 9). The glycoproteins are responsible for most of the envelope properties and can be resolved into two components by polyacrylamide gel electrophoresis or by chromatography (27, 28, 29).

The size of the viral glycoproteins are correlated with the transformed state of the cells, in which the virus is grown (9). The virus released from transformed cells contain bigger glycoproteins than the virus released from nontransformed cells. Furthermore, the difference in the size of the glycoproteins is found to be primarily due to the glycopeptide moieties, obtained by exhaustive digestion of glycoproteins with pronase. The glycopeptides of viruses released from transformed cells are bigger, by approximately 1200 daltons, than those of viruses released from nontransformed cells (9). However it is not known whether the increase in the size of glycopeptides is the result of the transformed state of the host cells or the result of specific viral gene products, e. g., some glycosyl transferase, of the sarcoma virus. The methylcholanthrene-transformed quail cell would provide a system for the study of this question since the transformed state of this cell line is not caused by the transformation genes of sarcoma viruses. Thus the effects of the host cells and of the viral oncogenes can be separated.

B. Oncogene and Protovirus hypotheses

Based on some sero-epidemiological and cell culture evidence, Huebner and Todaro suggested (3, 30) that the genomes of RNA tumor viruses, "virogenes", might be part of the cellular genome and are present in all vertebrate cells, albeit in a repressed form. The occurrence of cancer then might be due to the derepression of these endogenous viral genes (3, 30). Furthermore, these endogenous viral genes were considered to be the single unifying cause of cancer, as other types of carcinogens, for instance, chemical carcinogens, irradiation, or DNA tumor viruses might act through the activation of the endogenous RNA tumor viruses (3,30).

Indeed the presence of complete viral genomes in most of the normal chicken or mouse cells is indicated by nucleic acid hybridization (31, 32, 33) and by the presence of *gs* antigen (34) and by the chemical and physical induction of complete viruses from nearly every kind of chicken, mouse and rat cell (35, 36, 37). Therefore, it is quite likely that the viral genes are part of the cellular genes and are transmitted for generations without overt production of

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the endogenous viruses. However, the endogenous viruses induced thus far all belong to leukemia viruses which lack the transforming gene for fibroblasts. Thus, the presence of oncogene inside the cells which is responsible for the oncogenic transformation of the cells has yet to be proved.

On the other hand, Temin (4) hypothesized that RNA-DNA-RNA synthesis is a normal physiological process, and, in a rare event, might lead to the formation of genetic information for cellular transformation and viral production. The transduction of this oncogenic information by RNA tumor viruses might lead to the development of transforming sarcoma viruses.

Although these two hypotheses differ in the mechanism of the production of oncogenes and virogenes, they do agree in that the genetic information responsible for the transforming ability of the RNA tumor viruses is derived from the host cells. However, no successful attempts have been made to demonstrate such "oncogenes" inside the cells.

The following proposed project will attempt to detect such oncogenes incorporated into leukemia viruses while they are passaged in the chemically transformed cells. Since RNA tumor viruses replicate through a DNA intermediate, which is integrated into cellular chromosome (38), the recombination between viral genome and cellular transformation genes, activated by chemical carcinogens, is quite likely.

The electrophoretic analysis of RNA size and the two-dimensional fingerprinting of RNA sequences provide the sensitive techniques for the detection of any such recombination products. Even the virus which does not yet acquire transforming activity but already incorporated part of the transformation genes will be detected by these methods.

DETAILED RESEARCH PLAN

The following general methods for virus propagation will be used:

A. Cells: The cells to be used are the established quail cell lines derived from quail tumors caused by methylcholanthrene. Young adult Japanese quails (*Coturnix coturnix japonica*) were injected with methylcholanthrene intramuscularly, and tumors developed within one to two months. The tumors have been established in tissue culture as continuous lines by Dr. C. Moscovici and eight different tumor lines have been received from Dr. C. Moscovici and are now maintained in this laboratory.

For focus assays, the C/O and C/E fibroblasts derived from 11-day-old chicken embryos will be used.

B. Viruses: The quail cells are susceptible to avian tumor viruses of subgroups A, E and F and are resistant to the viruses of other subgroups (39, 40, 41). The leukemia viruses or transformation-defective viruses belonging to these subgroups, e.g., RAV-1 (Rous-associated virus), RAV-3, RAV-5, td PR RSV-A (a transformation-defective derivative of Prague strain Rous sarcoma virus), lymphoid leukemia virus RPL-12, RAV-60, or RAV-61 will be used to infect the quail tumor cells.

The quail tumor cells will be infected with various leukemia viruses at multiplicity of infection about 1. Daily medium changes are required. At day 4, the cells will be trypsinized and reseeded. This transfer increases the titer of viruses released into the medium. At day 7 or 8, the cells are confluent and will be labeled with radioactive precursors. The viruses will be purified from the medium by ammonium sulfate precipitation, sedimentation to a

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65%-20% sucrose interface, and finally equilibrium sedimentation. The virus has a density of 1.16-1.18 gm% (18). The RNA will be extracted with sodium dodecyl sulfate and phenol (18). The 70S RNA will be separated from 4S RNA by velocity sedimentation in sucrose gradients (18).

I. Do methylcholanthrene-transformed quail cells release virus particles?

Methylcholanthrene can transform mouse, rat, and hamster cells in vitro under a variety of conditions (42, 43). These chemically transformed cells have not been observed to produce any infectious tumor viruses (44). On the other hand, chemically induced tumors have been found in several cases to release tumor viruses (51, 52, 53). Furthermore, methylcholanthrene has also been used to induce the expression of the genome of endogenous RNA tumor viruses from normal chicken cells (35). The extent of induction ranges from production of the infectious virus particles to conversion of chicken cells from gs (-) to gs (+). It is therefore necessary to study the physiological states of the methylcholanthrene-transformed quail tumor cells which I plan to use in this project. The main questions are:

- (1) Are the quail tumor cell lines gs (+), i.e., contain the group-specific antigen of avian tumor viruses?
- (2) Do they produce infectious viruses?
- (3) If no, do they produce any physical particles resembling RNA tumor viruses?

The gs test has been established as a routine test for our primary chicken embryo fibroblast cultures in this laboratory. The method is as described by Vogt and Friis (54). It is a complement-fixation test for the detection of group-specific antigens in the cells. If the quail tumor cells turn out to be positive for gs antigen of avian tumor virus, while the normal quail cells from the same flock are negative, it would show that the normal quail cells contain the genome of avian viruses and the chemical carcinogen induces the partial expression of these gene functions.

The detection of focus-forming viruses released into the culture media will be done by the routine focus assay which has been developed by Rubin (45) and modified by Vogt (46). The focus assay will be performed on C/O chicken cells, which are susceptible to all the known strains of avian tumor viruses.

The detection of noninfectious viral particles will be done by radioactive labeling of cultured quail tumor cells. ^3H -uridine or ^3H -amino acids will be added to the culture medium, and the latter will be purified to detect any particles which have a density around 1.16 gm/ml and 1.18 gm/ml, the density of the RNA tumor viruses. These virus particles might represent expression of endogenous tumor viral genes.

Preliminary data suggest that, in one quail tumor cell line, a transforming virus is produced (Moscovici and Vogt, personal communication). In another cell line, release of noninfectious virus particles has been observed. These viruses will be studied in more details. Their RNA will be labeled with ^3H -uridine or ^{32}P , and analyzed by polyacrylamide gel electrophoresis, to see whether they contain class a or class b RNA, or RNA of even smaller size. It will also be digested with RNase T_1 and analyzed by two-dimensional electrophoresis and homochromatography to see whether it lacks any gene sequences. The

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protein structure of these particles will be studied by electrophoresis on 10% polyacrylamide gels to see whether they lack any protein components. This again will suggest that the genome of RNA tumor virus is present in the quail cells, and that the chemical carcinogen, methylcholanthrene, acts as an inducer for these viruses.

The quail tumor cell lines which produce infectious or noninfectious virus particles will not be used for the following study, since the endogenous virus is difficult to be distinguished from the superinfecting virus.

II. Serial passage of avian leukosis virus in the methylcholanthrene-transformed cell lines of the Japanese quail.

This experiment is designed to detect any change in the viral genome during serial passage of an RNA leukosis virus in methylcholanthrene-transformed cell lines. Transformed cell lines which do not produce any infectious or noninfectious virus particles will be infected with several strains of avian leukosis viruses. Most of the lines are gs (-), but gs (+) lines will also be used, if available. They will be studied separately. Since the purpose of this experiment is to detect acquisition of sarcomagenic potential by leukosis virus during the course of infection, only leukosis viruses will be used for the initial infection.

Quail cells have been shown to be susceptible to avian tumor viruses of subgroups A, E and F (39, 40, 41). They are relatively resistant to leukosis viruses of subgroups B, C and D (39). Subgroups E and F include endogenous viruses and induced leukosis viruses of chicken and ringnecked pheasant (40, 41). No natural sarcoma viruses have been found to fall into these two subgroups. It is not known whether this is due to the lack of recombination ability with host sarcoma genes in the endogenous viruses. Because of this peculiar feature of subgroups E and F, leukosis viruses and transformation-defective derivatives of sarcoma viruses belonging to subgroup A will be used first. Some examples of viruses to be used are Rous associated viruses, RAV-1, RAV-3, RAV-5, lymphoid leukosis virus, RPL-12 and the transformation defective derivative of Prague strain of RSV (td PR-RSV-A). The cells will be infected at a multiplicity of infection about 1. At day 7 or thereafter, when the cells are confluent, the culture medium will be collected and used for the virus stock to infect the new cells. These new cells will be regarded as the second passage virus. This virus will be used to infect the new cells ("third passage cells") again.

This cycle will be carried on. At the end of each passage, the cells will be labeled with ^3H -uridine or ^{32}P . The virus will be purified from the culture medium. The viral RNA will be analyzed in the following ways:

(1) polyacrylamide gel electrophoresis: All the leukosis viruses which will be used to infect the quail cell lines contain class b RNA (24). Presumably this RNA lacks some sarcoma gene sequences which are present in class a RNA of sarcoma virus (10). If the leukosis viruses acquire some sarcomagenic sequences from the host tumor cells during the course of transmission in methylcholanthrene-transformed cells, it will be expected that the size of RNA of the leukosis viruses will be bigger than class b RNA. The mechanism of integration of sarcomagenic information into leukosis viral genome might be through genetic recombination, which has been demonstrated to occur between endogenous virus and the superinfecting sarcoma virus (47). The genetic recombination between avian tumor viruses which results in the increase of viral genome size has also been demonstrated (Duesberg, personal communication). This recombination could be

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a stepwise process. The leukemia virus may pick up part of the transformation genes first. As the virus is serially passaged through the methylcholanthrene-transformed cell lines, the leukemia virus might acquire more sarcomagenic genes, and eventually their RNA reaches the size of class a RNA.

The superinfected tumor cell lines will be labeled with ^{32}P . The culture medium will be collected at three-hour intervals to minimize the degradation of viral RNA in the culture medium. The virus will be purified from the pooled culture medium. The 60-70S RNA of the virus will be mixed with the ^3H -uridine-labeled 60-70S RNA of the leukemia virus propagated in the normal quail embryo fibroblasts as a control. The RNA mixture will be heated at 100°C for 45 seconds (18) and the resulting 30-40S RNA will be electrophoresed in the 2% polyacrylamide gels. Any changes in the size of the viral RNA of leukemia virus propagated in the methylcholanthrene-transformed cell lines can be detected.

If the viral RNA increases in size, it will suggest that some cellular genes have been incorporated into viral genome. However, it is also likely that this is due to the amplification of part of the viral genome. To determine that cellular genes, particularly, sarcoma-specific genes, have been incorporated into the virus, oligonucleotide fingerprinting technique and focus assay of the virus will also be used.

(2) oligonucleotide fingerprinting: Sanger's two-dimensional fingerprinting method for RNA gave characteristic fingerprints for every strain of RNA tumor viruses (10; Lai, unpublished observation). This method involves digesting the RNA with RNase T_1 and then separating the resulting RNase T_1 -resistant oligonucleotides of the RNA by electrophoresis at pH 3.5 in the first dimension and homochromatography in the second dimension. About 20-25 larger oligonucleotides (10-50 nucleotides long), which account for about 2-3% of the total RNA, are resolvable, and their distribution patterns are the basis for characterizing the RNA. The rest of the RNA is degraded into oligonucleotides too small to be significant. I have used this method to compare the RNA of sarcoma viruses with that of leukemia viruses. It was found that all the genetic sequences in leukemia viruses are present in sarcoma viruses. Moreover, sarcoma viruses contain a few additional oligonucleotide sequences which are sarcoma-specific (10). These sarcoma-specific sequences could be used for identifying sarcoma genome.

Thus the ^{32}P -labeled 30-40S RNA of the leukemia viruses released from quail tumor cell lines and prepared according to the method described in the last section will be analyzed by the two-dimensional fingerprinting. If they incorporated the sarcoma-specific gene sequences from the host cells, the sarcoma-specific sequences identified previously (10) should appear in the fingerprints of the leukemia viruses released from quail tumor cell lines. However, since the RNA sequences identifiable by this method constitute only 2-3% of the total RNA sequences, this method of identifying RNase T_1 -resistant oligonucleotides should be less sensitive than the polyacrylamide gel electrophoresis of RNA. Failure to find the sarcoma-specific oligonucleotides does not rule out the possibility that part of the transformation genes is already integrated into the viral genome. The ultimate proof that the virus has incorporated the sarcoma-specific genes in the cells should come from the demonstration that the virus released from chemically transformed cells can produce foci on chicken embryo fibroblasts.

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III. Identification of leukosis viruses released from quail tumor cells.

If leukosis viruses containing bigger 30-40S RNA than class b RNA, or focus-forming sarcoma viruses are released from the chemically transformed quail tumor cells during the course of serial passages, further identification of viruses becomes necessary. There are two basic reasons:

- (1) Methylcholanthrene is known to induce the endogenous virus (35).
- (2) Infection of avian cells with some strains of avian tumor viruses can also induce endogenous viruses (40, 41).

Therefore the viruses released during the serial passages of the leukosis virus in the methylcholanthrene-transformed cells might be endogenous viruses, instead of the superinfecting viruses with incorporated host genes. Thus the viruses will be classified with regard to their host range, interference pattern and antigenicity (39). The classification techniques are established and all the virus strains are available in our laboratory. Since the superinfecting virus belongs to subgroup A and the one endogenous virus of quail cells isolated so far belongs to subgroup C (Vogt, personal communication), the two viruses can be easily distinguished. Furthermore, since every virus strain has its characteristic T_1 -oligonucleotide fingerprint (Lai, unpublished observation), the virus strains can be identified by studying its oligonucleotide fingerprint using the two-dimensional fingerprinting technique as described above. This will be used for further identification of the virus released from quail tumor cells. If it is the virus derived from the superinfecting leukosis virus, e.g., RAV-1 or td PR-A, it should share the subgroup-specific oligonucleotides with the virus grown in normal quail or chicken cells.

IV. The size of the glycoproteins and glycopeptides of the virus released from the chemically transformed cells.

The ^3H - or ^{14}C -glucosamine-labeled glycoproteins of RAV-1 or td PR-RSV-A released from the methylcholanthrene-transformed quail tumor cells will be compared with those of RAV-1 or td PR RSV-A released from the normal quail cells respectively. The glycoproteins will be coelectrophoresed in 5% polyacrylamide gels to compare their size. The glycopeptides will be obtained by exhaustive digestion of viral glycoproteins with pronase (1 mg/ml) and then analyzed by sephadex chromatography.

The glycoproteins and glycopeptides of RAV-1 or td PR-RSV-A released from the methylcholanthrene-transformed quail cells will also be compared with those of viruses released from RSV(RAV-1) or PR RSV-A transformed quail cells.

It has been shown that the viruses released from virus-transformed cells have bigger glycoproteins than those from nontransformed cells (9). If the glycoproteins and glycopeptides of viruses released from the chemically transformed cells have similar sizes to those of transforming sarcoma viruses, e.g., RSV(RAV-1) or PR RSV-A, it will suggest that the large size of the glycoproteins and glycopeptides in the viruses released from the transformed cells is the result of the transformed state of the host cells. It will also suggest that methylcholanthrene-transformed cells have similar physiological state to virus-transformed cells. On the other hand, if their sizes are similar to those of leukosis

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viruses, i.e., RAV-1 or td PR-RSV-A, grown in normal cells, it will suggest that the large glycoproteins and glycopeptides are coded for by the sarcoma viral genome.

When the viruses with incorporated cellular genes are obtained, their glycoproteins and glycopeptides will also be studied in the same way to see whether there is any change in their sizes.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

The space available to me includes 600 square feet of laboratory space, kitchen space for glassware washing, animal room and secretarial office space which is provided in conjunction with the Department of Microbiology office.

The Department of Microbiology has provided me with part of high-voltage electrophoresis apparatus, including one high voltage power supply and one electrophoresis tank, one Wedco incubator and one refrigerator. I also have access to one Sorvall centrifuge, one Beckman centrifuge, 4 Spinco ultracentrifuges equipped with SW 50.1, SW41, SW27 and SW25.2 rotors, 2 Packard scintillation counters, one Gilford spectrophotometer and one darkroom.

11. Additional facilities required:

1. Inverted Microscope for daily examination of tissue culture cells (requested in this application.

2. Revco Deep Freezer. Savant electrophoresis tank, Spinco SW65 rotor, Corning still, Fraction collector: requested from Public Health Service and American Cancer Society. Still pending.

12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

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3a

12. Biographical Data

Personal Information:

Name: Michael Ming-Chiao Lai
 Business Address: Department of Microbiology
 University of Southern California
 School of Medicine
 2025 Zonal Avenue
 Los Angeles, California 90033
 A.C. 213-225-1511, Ext. 308
 Business Phone:
 Date of Birth: **REDACTED**
 Place of Birth: **REDACTED**
 Citizenship:
 Sex: Male
 Marital Status: **REDACTED**

Education:

REDACTED
 National Taiwan University College of Medicine, Taipei
 M. D. (Bachelor of Medicine)
 Intern, National Taiwan University Hospital
 Internist Officer, Chinese Marine Corps
 Department of Molecular Biology and Virus Laboratory
 University of California, Berkeley
 Ph. D. in Molecular Biology

Professional Background:

1973 to present	Assistant Professor, Department of Microbiology University of Southern California School of Medicine
1973 (January-June)	Postgraduate Research Molecular Biologist Department of Molecular Biology, University of California, Berkeley
1969-1972	Research and Teaching Assistant Department of Molecular Biology and Virus Laboratory University of California, Berkeley
1968-1969	Internist Officer Chinese Marine Corps
1968	Passed doctor's qualification Educational Council for Foreign Medical Graduates
1967-1968	Intern National Taiwan University Hospital
1961-1967	Medical Student National Taiwan University College of Medicine
1965, 1964	Summer Research Student Department of Biochemistry, National Taiwan University

Honors:

M. D. - Summa cum laude (1968)

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13. Publications:

- Lai, M. C. (1968) A study of sex chromatin in normal Chinese newborns. M.D. thesis. National Taiwan University College of Medicine.
- Lai, M. M. C. and P. H. Duesberg (1972). Adenylic acid-rich sequences in RNAs of Rous sarcoma virus and Rauscher mouse leukemia virus. *Nature* 235: 383-386.
- Lai, M. M. C. and P. H. Duesberg (1972). Differences between the envelope glycoproteins and glycopeptides of avian tumor viruses released from transformed and from nontransformed cells. *Virology* 50: 359-372.
- Lai, M. M. C. (1973). On the structure of RNA tumor viruses. Ph.D. dissertation. University of California, Berkeley.
- Lai, M. M. C., P. H. Duesberg, J. Horst and P. K. Vogt (1973). Avian tumor virus RNA: A comparison of three sarcoma viruses and their transformation defective derivatives by oligonucleotide fingerprinting and DNA-RNA hybridization. *Proc. Nat. Acad. Sci. U.S.* 70: 2266-2270.
- Lai, M. M. C., J. Horst, and P. H. Duesberg (1973). The RNA of non-defective avian sarcoma viruses and corresponding transformation defective segregants: A comparison by oligonucleotide fingerprinting and RNA-DNA hybridization. *Virus Research*, (2nd ICN-UCLA Symposium on Molecular Biology) (Eds. F. Fox and W. Robinson), p. 345.
- Duesberg, P. H., E. Canaani, M. M. C. Lai and P. K. Vogt (1972). News and reviews on avian tumor virus RNA (Abstract). IV. Lepetit Colloquium Cocoyoc, Mexico.
- Duesberg, P. H., E. Canaani, K. v. d. Helm, M. M. C. Lai and P. K. Vogt (1973). News and reviews on avian tumor virus RNA. In: Possible Episomes in Eukaryotes (IV. Lepetit Colloquium) (Ed. L. G. Silvestri). In press.
- Duesberg, P. H., P. K. Vogt, J. Maisel, M. M. C. Lai and E. Canaani (1973). Tracking defective tumor virus RNA. *Virus Research* (2nd ICN-UCLA Symposium on Molecular Biology) (Eds. F. Fox and W. Robinson), p. 327.
- Maisel, J., V. Klement, M. M. C. Lai, W. Ostertag and P. H. Duesberg (1973). Ribonucleic acid components of murine sarcoma and leukemia viruses. *Proc. Nat. Acad. Sci. U.S.* 12: 3536-3540.
- Duesberg, P. H., M. M. C. Lai and J. Maisel (1973) Tumor virus RNAs and tumor virus genes. In "Modern Trends in Human Leukemia - Biological, Biochemical and Virological Aspects". (Eds. R. Neth, R. Gallo and F. Stohman). J. F. Lehmanns Verlag München, Germany. In press.

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14. First year budget:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s)
even if no salary requested)

% time

Amount

1. Michael M. C. LAI, Principal Investigator (74-75)

40%

Lai, 1/9 of annual salary - summer supplement

100%

(see Justification, 4a)

2. Research Assistant (to be recruited)

9 months 50%

3 months 100%

Technical

Lab Helper (to be recruited)

20 hours/week

subtotal

12% S and W F. B.

Sub-Total for A

B. Consumable supplies (by major categories)

Radioisotopes

5100

Sera, media, chemicals

2300

Scintillation vials, and counting fluids

1600

Disposable tissue culture supplies

1100

Glassware

600

Sub-Total for B

10,700

C. Other expenses (itemize)

Service contract; repair

1065

Xerox

300

Telephone

200

Publication costs

250

Sub-Total for C

1,815

Running Total of A + B + C

23,398

D. Permanent equipment (itemize)

1. Zeiss Inverted Microscope

1250

2. Mettler top loading balance

980

3. Lakeside Carts

175

4. Refrigerator

360

5. Virtis pipette dryer

250

Sub-Total for D

3,015

E. Indirect costs (15% of A+B+C)

E

3,510

Total request

29,923

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	2	10,400	3,577	1,050	4,315	34,131
Year 3	2	11,696	4,006	----	4,771	36,530

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Justification of Budget:

Salaries: The salary of the Principal Investigator represents a summer salary as per University policy. The University of Southern California is on a nine-month salary scale. The Principal Investigator will not take vacation during the summer months. Two ninths of annual salaries were requested from Public Health Service. The salaries requested in this application represents one ninth of the nine-month salaries. During the second and third years, the laboratory helper will become full-time. Increases in salaries are based on the past fiscal average merit increases as per University policy.

Consumable supplies: Radioisotopes - most of the experiments proposed in this project involves the use of radioactive chemicals. The following figures are rough estimates of major radioisotopes to be consumed in a year on this project:

^{32}P : 1,000 mCurie	(\$1200)
^3H -uridine: 100 mCurie	(900)
^3H -glucosamine: 30 mCurie	(1500)
^{14}C -glucosamine: 3 mCurie	(1500)

Scintillation vials and counting supplies: Virus purification, RNA preparation by sucrose gradients, polyacrylamide gel electrophoresis, column chromatography all require counting in a liquid scintillation counter. About 800 vials per month will be used for this project.

Glassware: To avoid contamination by RNase, most of the biochemical glassware to be used will be disposable.

Plasticware, sera, media and chemicals: This project involves the use of large amounts of tissue culture.

Equipment: Inverted microscope: necessary for daily examination of tissue culture cells. Presently no microscope is available to me in the laboratory.

Mettler balance: necessary for various measurements in biochemical studies.

Carts: necessary for various purposes in the laboratory.

Refrigerator: Presently there is only one refrigerator in the laboratory. One more refrigerator is required for storing tissue culture media and biochemical materials separately.

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16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE			
Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
1) Structure and Replication of Rous Sarcoma Virus	Institutional Grant from American Cancer Society	\$3,000	Aug. 15, 1973 - Apr. 30, 1974
2) Structure and Replication of Rous Sarcoma Virus	California Division of American Cancer Society (Special grant #656)	\$10,000	Oct. 1, 1973 - Sept. 30, 1974

PENDING OR PLANNED			
Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Structure and Replication of Rous Sarcoma Virus	1) Public Health Service Dept. of Health, Education and Welfare (CA 16113-01)	\$159,307	Pending (May 1, 1974 - Apr. 30, 1977)
	2) American Cancer Soc.	\$185,650	Pending (July 1, 1974 - June 30, 1977)
	Only one of the above, if awarded, will be accepted.		

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Checks payable to

University of Southern California

Mailing address for checks

University Park

Los Angeles, Ca. 90024

Principal investigator

Typed Name Michael Ming-Chiao LAI

Signature *Michael Ming-Chiao Lai* Date 1-25-74

Telephone 213 225-1511 x 308
Area Code Number Extension

Responsible officer of institution

Typed Name Z. A. Kaprielian

Title Vice Pres. Academic Administration and Research

Signature *Z. A. Kaprielian* Date

Telephone 213 225-1511 x 432
Area Code Number Extension

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#766ARI - LERNER

1003545212

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

February 13, 1974

Grant application No. 766AR1

CANCER

To: The committee comprising Drs. Gardner, Huebner and Meier

Subject: Richard A. Lerner, M.D. Scripps Clinic and Research Foundation, LaJolla
Renewal application No. 766AR1
"Studies on Persistent Viral Infection"

History

CTR has supported this study since 1970.

In March 1973, the SAB approved a two-year program at a level of \$47,385. Subsequently Dr. Lerner requested and was awarded a supplement of \$5,000, making his current level \$52,385.

Request

Application No. 766AR1 requests \$65,378.

Documents Submitted (Enclosed)

1. Application dated 1/28/74, incorporating summary progress report with VII tables.
2. CV of Chi-Hung Siu, new staff member.

Comment

Reprints or preprints of recent papers have been provided and will be forwarded if you wish.

Although Dr. Lerner had a "commitment" for one year more at approximately \$47,000, his request has now drifted so far from this figure that it appears necessary to present the enclosed application to the full SAB in March.

FWN:gh
Encls.

FWN
F.W.N.

1003545213

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

FEB 8 1974

Application For Renewal of Research Grant

(Use extra pages as needed)

First Renewal ☐

Second Renewal ☒

Date: 1/28/74

1. Principal Investigator (give title and degrees):

Richard A. Lerner, M.D., Associate Member

2. Institution & address:

Scripps Clinic and Research Foundation
476 Prospect Street
La Jolla, California 92037

3. Department(s) where research will be done or collaboration provided:

Department of Experimental Pathology

4. Short title of study:

Studies on Persistent Viral Infection

5. Proposed renewal date: July 1, 1974

6. How results to date have changed earlier specific research aims:

7. How results to date have changed earlier working hypothesis:

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8. Any additional facilities now required? Describe briefly:

no additional facilities required

9. Any changes in personnel? Append biographical sketches of new key professional personnel:

Addition of Chi-Hung Siu

10. Append outline of experimental protocol for ensuing year.

11. List publications or papers in press resulting from this or closely related work. (append reprints or manuscripts not previously sent).

See attached list

1003545215

12. Summary progress report (append in standard form as separate document, unless recently submitted).

13. Budget for the coming year:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s)
even if no salary requested)

	% time	Amount
Richard A. Lerner	50%	-0-
Frank J. Dixon, Consultant	--	-0-
David Kohne	100%	-0-
Fred Jensen	100%	-0-
Alvaro Puga	100%	10,480
Stephen J. Kennel	50%	-0-
Bert C. Del Villano	100%	-0-
Chi-Hung Siu	100%	8,000

Technical

Catherine Morris, Technician	100%	9,000
Part-time services of: secretary, animal caretakers, photographer, glass washers, histology technician, EM technician, machinist & electronic repairman		7,200

Sub-Total for A 34,680

B. Consumable supplies (by major categories)

Tissue culture media, glassware and plasticware	8,000
Isotope labeled compounds	5,000
Liquid scintillators and vials	5,000

Sub-Total for B 18,000

C. Other expenses (itemize)

Photography, histology, part-time use of electron microscope	4,170
---	-------

Sub-Total for C 4,170Running Total of A + B + C 56,850

D. Permanent equipment (itemize)

None

Sub-Total for D -0-E 8,528

E. Indirect costs (15% of A+B+C)

Total request 65,378

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14. Other sources of financial support: _____
List financial support from all sources, including own institution, for this and related research projects.

List financial support from all sources, including own institution, for this and related research projects.

Source
(give grant numbers)

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Studies on persistent viral infection	Council for Tobacco Research #766A	\$47,385	7/1/73-6/30/74
Molecular structure of the immunoglobulin receptor in continuous cultures of diploid human lymphocytes	National Science Foundation GB 34296	\$65,000	6/1/72-11/30/74
Immunopathology of virus infection (Salary - RAL)	NIH Career Development Award AI-46372	approx. \$125,000	7/1/70-6/30/75

Source
(give grant numbers)

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

Principal investigator

Signature Richard A. [illegible] Date

Telephone 714/459-2390, ext. 470

Responsible officer of institution

Typed Name Edmund L. Keeney, M.D.

Title President and Director

Signature Edward A. Keenan Date 2/1/74

Telephone 714/459-2390, ext. 357

Sports Clinic and Research Foundation

Mr. O.K. Kincaid, Jr., Controller
Scripps Clinic and Research Foundation
476 Prospect St., La Jolla, Calif. 92037

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10. Outline of Experimental Protocol

A. Introduction

This project continues to offer a multidisciplinary study of oncornaviruses, and the nature of their interaction with their host which may lead to the formation of tumors or to the development of immunologic diseases or both. The disciplines involved are virology, molecular biology, biochemistry and immunopathology. The immunologic emphasis in this work relating both to the immunopathologic consequences of oncornavirus infection and to the use of immunologic markers of viral presence are perhaps a unique aspect of this proposal. A better understanding of the immunologic consequences of oncornavirus infection is needed if we are to understand viral oncogenesis and host defense mechanisms, and to achieve effective therapeutic and/or prophylactic immunotherapy and also to avoid the possible immunopathologic complications of such therapy. The studies proposed are designed to contribute toward such an improved understanding.

B. Objectives and Scope

This project focuses on several interrelated aspects of the oncogenic and immunopathologic consequences of oncornavirus infection with particular emphasis on the situation in NZ mice. The first of these is the possible role of SLV, an oncornavirus(es) obtained from NZB lymphoblast cultures, in the genesis of spontaneous tumors and immunologic diseases of the NZ mice. Second, using SLV as a model oncornavirus, we are attempting to find if and how in vivo infection of a variety of murine and rat strains with this agent will cause tumors and immunologic diseases. Third, we plan to isolate and characterize the glycoprotein surface antigens of oncornaviruses and use this antigen as one of the yardsticks in evaluating the host-virus relationship. Fourth, we are analyzing the number and nature of the viruses produced by continuous thymoblast lines derived from NZB and related mice. Fifth, we propose to correlate a variety of virion coded or induced phenotypic markers of infected lymphoblasts with their oncogenic or immunologic potential in vivo. Sixth, we will study the incorporation of exogenous nucleic acids into oncornaviruses and other RNA viruses and attempt to determine whether this process may play a role in the induction of "autoimmune" responses following infection with their viruses. And seventh, we are searching for human C type viruses in the placentas of humans with lupus erythematosus.

The concept of immunological tolerance to viruses causing persistent infection after intrauterine or neonatal infection was first challenged seven years ago by the demonstration that mice vertically infected with LCM did in fact make an immune response to the virus and that this response caused a fatal immunologic disease (Oldstone and Dixon, *Science*, 158:1193, 1967; Oldstone and Dixon, *J. Exp. Med.* 129:483, 1969). Since then numerous similar reports have identified immune responses to many persistent viruses including the oncornaviruses (Oldstone, Aoki and Dixon, *PNAS*, 69:134, 1972) with associated immunological diseases of varying severity. The manifestations of these immune responses, i.e., antibodies and sensitized cells, can serve as indicators of the presence of such infections and in the case of oncornaviruses provides one of the best and at times the only evidence

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of their existence. The fact that antibody responses to oncornaviruses may be associated with particular immunologic diseases such as immune complex nephritis, or more specifically, lupus type nephritis, prompts a search for oncornaviruses in patients with these conditions. Several laboratories including our own have identified the initiation of antinuclear antibody (ANA) formation in animals as a result of oncornavirus infection further linking these viruses to human immunologic disease and suggesting that nuclear antigens may be presented in a particularly immunogenic form as a result of oncornavirus infection. Thus, the pathologic package offered by oncornavirus infection may include tumors for the least immunologically responsive host and immunologic disease for the most responsive with appropriate combinations for those in between. The experiments described below are designed to determine whether this hypothesis is true and if so how the virus acts to initiate either tumors or self destructive immune responses and what factors in the host determine the pathological outcome of such an infection.

C. Background (see Progress Report)

D. Experimental Procedures

1. Effects of neonatal infection of mice and rats with SLV. We intend to complete our ongoing study of the oncogenic and immunopathologic consequences of neonatal infection of mice and rats with SLV as described in the accompanying progress report. Our purpose of this initial screening approach is to identify strains of mice or rats which react oncogenically or immunopathologically in particular ways to SLV infection, thus providing us with host-virus combinations which may be studied for the particular factors predisposing to specific disease states. Although this study has been underway for only a few months, we already have several promising leads which appear worthy of follow-up.

a. Factors regulating serum gs levels in animals infected as neonates with SLV. The 2-3 month serum gs levels of all strains of SLV infected mice and rats are 10 to 60 times greater than normal, except for the NZB x W and NZW x B mice in which gs levels are not significantly elevated. The low serum gs levels in the NZ hybrid mice could be the result of either the failure of SLV to infect or multiply in these animals or the operation of a suppressive immunologic response which was effective in rapidly removing the gs and perhaps other viral antigens from the circulation. In order to test these hypotheses, we will conduct a combination of in vivo and in vitro studies. The tissues (spleens, thymus, lungs and kidneys) of SLV infected NZ hybrid mice taken at 1, 3 and 6 months of age will be assayed by quantitative immunochemical procedures for gs and GP-70 SLV antigens in order to determine whether these viral products are being produced in the tissues. These results from the NZ hybrids will be compared with results of similar observations made in the high serum gs strains, thereby providing some idea of relative rates of oncornavirus synthesis in the different strains. In vitro studies will include

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the growth of primary cultures of spleen, thymus, lungs and kidneys from SLV infected NZ hybrids and from SLV infected high serum gs strains and the determination of the amount of infectious virus formed. These two procedures should indicate whether SLV can and does infect and replicate in the NZ hybrids as well as in other strains. If it can the results would suggest that the low serum gs levels in the former are the result of immune elimination of the viral antigens by circulating antibody and this would fit with the well recognized immune complex disease from which these mice suffer.

b. The basis for resistance of NZ hybrids and C57 Black/6J mice to SLV induced tumors. All the strains of mice and rats infected with SLV in our screening study except the NZ hybrids and to a lesser extent the C57 Bl/6J develop a high incidence of lymphoma and/or leukemia. The means by which these two kinds of mice respond to and control the oncogenic effects of SLV infection are probably different in view of the widely differing gs levels they have. In order to get some idea of the virus-host relationship in these resistant strains we will check them at 1, 3 and 6 months for neutralizing antibody to SLV and also see whether the virus present in each has the same biological behavior. To determine the latter we will isolate virus from SLV infected NZ hybrids, C57 Bl/6J and from one or two high leukemia strains and titrate these isolates by X-C test in vitro and inject them into neonatal Balb/c mice to determine their leukemogenicity in vivo. These two lines of study should give some idea of the nature of the resistance of these mice, i.e., whether they are resisting an oncogenic virus or whether the virus replicating in them is not oncogenic.

c. The nature of ANA formed by mice responding in different ways to SLV infection. High levels of ANA are observed in two groups of mice responding quite differently to SLV infection. In the NZ hybrid strains there is considerable ANA production and a high incidence of severe glomerulonephritis, low incidence of lymphoma and low levels of circulating gs. In the C57 Black/6J and C57 Black/10J there is a high level of ANA with little or no glomerulonephritis, a low to moderate incidence of lymphoma but high circulating gs levels. The immunologic specificity of the ANAs made by these two groups of animals will be compared by quantitative and qualitative immunochemical procedures. The spectrum of antinuclear antibodies in each of these types of sera will be determined by measuring the amount of antibody capable of reacting with double stranded DNA, with single stranded DNA, with double stranded RNA and single stranded RNA by modifications of the Farr technique which are currently employed for these determinations in our laboratory. In addition by absorption and blocking procedures using both antigen binding tests and fluorescent antibody determinations as end points we can determine the approximate amount of antibodies in these sera against histones, nucleo-protein, and the SM nuclear antigen. These studies should provide valuable leads as to the immunopathogenicity of various ANAs. They may also allow us to learn whether the immunogenic challenges presented by SLV in the different strains of mice are different or whether the genetic backgrounds of the mice dictate different responses to similar antigenic challenges.

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d. The immunologic basis for the glomerulonephritis of SLV infection associated with high and low ANA responses. A high incidence of glomerulonephritis was observed in both the New Zealand hybrid strains associated with a high ANA response and in the C57 brown mice with a low ANA response. Since the pathogenetic mechanism in this form of glomerulonephritis is almost certainly antibody mediated we plan to compare the antibodies found in the glomeruli of the diseased kidneys of these two kinds of mice. The kidneys from approximately 30 to 40 nephritic mice of each kind will be pooled, homogenized, washed repeatedly in cold saline and then the residual immunoglobulin eluted at pH 3.2 in citrate buffer (J. Exp. Med. 127:507, 1968). This treatment removes the Ig bound immunologically in the kidney and allows one to determine the specificity of the antibodies involved in the nephritogenic process. The eluates will be neutralized and concentrated to a convenient volume. The concentration of Ig in the eluates can then be determined by radioimmunodiffusion and aliquots of the eluates can be absorbed by various insoluble nuclear and viral antigens after which the Ig concentration is again determined and from the difference in the pre and post absorption concentrations, the percent of immunoglobulin eluted which is reactive with the antigens employed in absorption can be obtained. With the particular strains selected by our screening procedure one might expect to find quite different antigen-antibody systems involved in the nephritogenic process in the respective strains. Such observations would help define the nature of nephritogenic immune complexes induced by oncogenic viral infections and by the nature of the antigens involved might tell us a good bit about the role of viral infection in antigen liberation. These observations would also go a long way toward determining whether the immunologic renal disease produced by infection with these viruses is antiviral in nature or "auto-immune". High levels of ANA are observed in two groups of mice representing

2. Influence of immunosuppression and immunotherapy on the course of spontaneous and induced SLV infection and oncogenesis.

Of all the strains of mice observed in our screen to date, only the NZ hybrids and to a lesser degree the C57 Black/6J mice appear highly resistant to the development of lymphoma. We propose to try to ascertain the importance of the immune response in these animals in the resistance to lymphoma. Both the New Zealand hybrids and the C57 Black/6 mice will be infected with SLV at birth as in the screening study and then as soon as possible put on continuing immunosuppressive doses of Imuran or comparable agents. NZ hybrid and C57 Black/6 mice uninoculated with SLV will be similarly subjected to immunosuppression and serve as controls. These animals will be followed for the development of tumors, glomerulonephritis and ANA. The conduct of these experiments will be similar to that for the screening experiment now underway (see Progress Report). The degree of suppression of the cellular and humoral immunologic processes will be tested by appropriate antigenic challenge during the course of the experiment. If as seems likely we are able to modify the course of SLV infection by this kind of immunosuppression and for example observe a high incidence of lymphoma, it would suggest that the immune response or something else affected by Imuran

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was playing a significant role in the repression of tumors in the unsuppressed animals. In addition, if we observe tumors, particularly lymphomas, in the immunosuppressed but uninfected mice of either strain it would suggest that these animals do carry potentially oncogenic viruses which are held in check apparently at least in part by immunologic mechanisms.

If such an immunosuppressive regime were effective in inducing tumors in SLV infected animals otherwise resistant to oncogenesis, the suppressed animals might provide a useful model for immunologic manipulation of developing tumors. Termination of immunosuppression, with or without passive transfer of antibodies or syngenic sensitized cells, could be used in attempts to control the growth of a "spontaneous" tumor. In such a therapeutic situation the course of ancillary immune responses such as ANA and the diseases such as glomerulonephritis associated with them could also be observed to see whether such an untoward immunopathologic development would occur in the course of experimental immunotherapy.

Immunotherapy of viral oncogenesis can also be attempted in mice such as Balb/c and SWR/J which have a high incidence of lymphoma after neonatal SLV infection. Since adult mice of these strains are resistant to the oncogenic effects of either SLV infection or transfer of malignant SLV producing lymphoblasts, transfer of syngeneic adult lymphoid cells to neonatally infected mice can be attempted at various times before and after appearance of the tumor in order to observe the efficacy of such immunotherapy and the possible development of immunopathologic complications.

3. The effect of neonatal oncornavirus infection on the immunologic properties of mice. Since oncornavirus infection produces a number of immunologic responses in non-New Zealand mice which are characteristic of the immunologic disease picture seen in unmanipulated New Zealand mice, and since it has been claimed that there is a deficiency in the cellular immune mechanisms and an associated hyperresponsiveness in the humoral immune mechanisms of unmanipulated New Zealand mice, it would be interesting to determine whether the oncornavirus infection in non-New Zealand mice produce changes in the immunologic system making them similar to New Zealand mice. To do this we plan to infect neonatal Balb/c x NZB hybrids, C57 Brown and C57 Black/6 mice with SLV. Their cellular and humoral immunologic responsiveness will be tested periodically by attempted induction of immunologic tolerance, by *in vitro* responsiveness to sheep red blood cells using the Jerne plaque technique, and by the humoral and cellular responses to various antigens as determined by primary antigen binding tests and by lymphocyte transformation or MIF production. If oncornaviruses such as SLV and Moloney which do induce autoimmune type responses in mice also produce the same changes in the immune system that are found spontaneously in New Zealand animals, it would suggest that the primary effect of these viruses on the lymphoid system is important in producing the aberrant responses. If on the other hand, the viral infections do not produce such a functional change in the lymphoid system, it would suggest that these viral infections operate via the release and/or abnormal presentation of certain endo-

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ogenous and/or exogenous antigens. While there have been a number of reports of alterations in the immune responses of animals infected with oncogenic viruses, there has not been a study carried out designed to show whether these immunologic alterations might be important in the development of immunologic disease.

4. Isolation and characterization of the glycoprotein surface antigens of oncornaviruses and leukemic cells. Virus induced cell surface antigens of leukemia cells are of importance as they represent targets for the immune defense of the host. These antigens may represent markers indicating the degree of expression of an oncogenic viral genome and they may also affect cell membranes in a way which leads to the altered behavior of transformed cells. A review of the extensive serological studies of Gross virus antigens has been presented by Aoki et al. (Aoki, T. et al., J. Virol. 10:1208, 1972). Although these approaches have led to advances in the study of tumor antigens, they have certain limitations, as data from these serologic techniques bear no direct relation to antibody interaction with specific membrane components. In order to gain a better understanding of the complex role of viral envelope antigens in oncogenesis, it is necessary to isolate them and to determine their immunochemical and molecular characteristics.

a. Objectives and scope. Our major aims are 1) to isolate sufficient quantities of viral envelope antigens required for their thorough immunochemical and chemical characterization and 2) to utilize these well defined reagents to produce antibody suitable to assess the biological relevance of these viral antigens for the overall problem of carcinogenesis. Specifically, we will assess the role of these antigens in viral infectivity by determining their capacity to produce specific neutralizing antibody. We will also attempt to evaluate to what extent antigenic determinants are shared between viral envelope antigens which differ widely in their tropisms as we utilize two B- and two N-tropic viruses, respectively. Ultimately, it will be of key importance to determine whether reagents developed during this study can be utilized to inhibit tumor growth in the animal model system.

b. Background. Soluble hemagglutinating surface antigens were isolated by enzyme treatment of several murine leukemia viruses (Whitter, R. et al., Virology 54:330, 1973). These antigens were able to inhibit potent neutralizing antisera directed against tween ether degraded virus particles. Removal of these surface antigens by bromelain treatment resulted in complete loss of virus infectivity. These antigens were shown to have a molecular weight of 93,000 and appeared to be glycoprotein in nature. The isolation of Rauscher murine leukemia virus polypeptides containing an interspecies antigen was recently reported by Strand and August (Strand M. and August, J.T., J. Biol. Chem. 248:5627, 1973.) These investigators utilized freezing and thawing together with sonication to solubilize these antigens which upon further purification by ion-exchange chromatography yielded two components upon SDS-acrylamide gel electrophoresis with molecular sizes of 69 and 71,000, respectively (GP 69-71). Radioimmuno-

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assay with anti-feline virus serum revealed that both polypeptides contained the interspecies determinants. It is believed that these antigens are virus coded due to the selective nature of the interspecies antigenic determinants and their absence in uninfected cells.

A glycoprotein of 70,000 molecular weight (OSA) was detected on an oncogenic C-type virus, Scripps leukemia virus (SLV) produced by a lymphoblastoid cell line from an NZB mouse (Kennel, S. J. et al., Virology 55:464, 1973). By selective radiolabeling, OSA was shown to comprise 0.1% of total cellular amino acids and 10% of the cellular glucosamine. This protein was found to react with sera which neutralized Moloney, Kisten, Rauscher, AKR and Scripps viruses and is believed to be the antigen which may be involved in virus neutralization.

c. Experimental procedure--production of antibody against SLV GP 69-71.

To be able to develop a meaningful radioimmunoassay for SLV GP 69-71, we will utilize the purified antigen on hand to produce specific antibodies in rabbits. For this purpose we will make bi-weekly injections in complete Freund's adjuvant (CFA) initially by injecting the material in the lymph nodes to be followed by bi-weekly subcutaneous injections in CFA. We will ascertain whether any neutralizing antibody thus produced by testing the rabbit sera in the XC-cell plaque assay as described by Rowe et al. (Rowe, W. P., Virology 42:1136, 1970).

Development of radioimmunoassay for SLV GP 69-71. We will utilize the SLV GP 69-71 isolated thus far to develop a radioimmunoassay essentially as described by Hunter (Hunter, W. M., in Handbook of Exptl. Immunology, Weir, P. M. ed., pp. 608-642, 1967, F. A. Davis, Philadelphia). This assay will permit the quantitative analysis of viral antigens and will be utilized to measure the progress of viral antigen isolation, purification and chemical characterization. The antigenic profile of GP 69-71 antigens isolated from SLV, RLV, wild mouse and AKR leukemia viruses will be compared by use of the competitive radioimmunoassay. To determine whether GP 69-71 is not just a cell membrane component, it will be necessary to test whether either uninfected host cells or those infected with another membrane maturing virus, e.g., VSV, also contain GP 69-71.

Assessment of GP 69-71 on wild mouse (1504E) and AKR virus. To determine whether these two viruses contain GP 69-71, we will first assess whether neutralizing antibody produced against SLV GP 69-71 can be inhibited by these two viruses. Once we thus establish the presence of GP 69-71 on wild mouse and AKR viruses, we will attempt to isolate these antigens essentially by the same methods developed by us for the isolation of SLV GP 69-71.

Antigenic comparison of GP 69-71 of different viruses. We will determine whether envelope antigen from SLV, RLV, AKR and wild mouse viruses (1504E) share common antigenic determinants. For this purpose we will absorb neutralizing

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antisera directed against purified GP 69-71 of these above viruses with cultured NZB fibrosarcoma cells which are essentially free of SLV but possess GP 69-71 antigens. The residual neutralizing capacity of these absorbed antisera against intact viruses will then be determined. We will also utilize competition radio-immunoassays and gel diffusion analyses to compare antigenic profiles of the GP 69-71 components of different viruses.

Chemical characterization of SLV GP 69-71. We will first evaluate the degree of charge homogeneity of the isolated antigens. To this end we will individually cut out these components from SDS - acrylamide gels, remove the SDS by extensive dialysis against 10M urea, and analyze the antigen bands on urea-acrylamide gels for charge heterogeneity under different conditions of pH and pore size.

If there exists any charge heterogeneity we will determine whether this is due to either carbohydrate, i.e., sialic acid and/or amino acid composition. We will thus treat GP 69 and GP 71 components with neuraminidase and then evaluate their electrophoretic profiles in urea-acrylamide gels.

To ascertain whether irrespective of any charge differences, GP 69 and GP 71 contain a different peptide composition, we will analyze them by tryptic peptide maps. To this end we will utilize similar techniques as those used by Moss (Moss, B. et al., Virology 55:143, 1973). Thus, we will label the NZB cultured cells in situ with a mixture of ^{14}C amino acids or with ^{14}C glucosamine and isolate GP 69 and GP 71 from semi-preparative (1.6 mm) acrylamide gels by cutting out the two bands which contain these antigens. The proteins will be eluted with SDS, precipitated in TCA, dissolved in performic acid, lyophilized and then digested with trypsin. Peptide maps will be made on cellulose thin layer sheets and the patterns visualized with x-ray film. This approach should make it possible to evaluate to some extent the structural differences in these two molecular size classes of viral surface antigens. Labeling with ^{14}C hexosamine will tell us whether or not structural differences between the two moieties are located in their glycopeptide components. We will also determine the amino acid composition of SLV GP 69-71 and of the individual GP 69 and GP 71 components, respectively. A special effort will be made to carry out careful performic acid oxidations to ascertain the number of cysteic acid residues to give us an idea of the disulfide bridge content of these molecules. If disulfide bridges are present, we will completely reduce and carboxamidomethylate the components and then assess by acrylamide gel electrophoresis in the presence of either SDS or urea whether the antigenic moieties consist of one or more polypeptide chains.

An effort will be made to establish the carbohydrate profile of SLV GP 69-71. For this purpose we will utilize gas chromatographic analyses already ongoing in our laboratory. Depending on carbohydrate composition and sialic acid content, we will utilize either neuraminidase or specific purified glycosidases (on hand in our laboratory) to digest these antigens while monitoring their remaining carbohydrate residues by gas chromatography. This approach

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should allow us to determine the role which carbohydrate moieties play in expressing the antigenicity of GP 69-71. In particular, it would be of interest if there would be any change in the inhibitory capacity of these antigens in virus neutralization assays following the removal of carbohydrate groups or whether antigens treated in this manner vary greatly in their reactivity in the radioimmunoassay.

Finally, we will attempt to determine the minimal molecular size required for GP 69-71 to express its antigenic activity. To this end we will use controlled proteolysis with insolubilized trypsin at non-optimal pH for digestion to obtain relatively large protein fragments. Such moieties could then be tested in the radioimmunoassay or assayed for their capacity to inhibit neutralizing antibody.

5. Studies on the number and nature of viruses produced in continuously growing thymocyte lines established from the New Zealand mice. From the work cited above, as well as the studies of others, it is clear that oncornaviruses may play a role in the pathogenesis and etiology of the autoimmune disease of the New Zealand mice. However, since all of the studies, including our own, have been carried out with a complex mixture of oncornaviruses, it is of extreme importance to characterize in as much detail as possible the nature of the viruses responsible for the various pathological processes observed when they are injected into newborn mice.

a) A single virus could cause more than one disease such as tumors and autoimmunity in a single mouse.

b) A single virus might cause one disease in one strain of mice and a different disease in another strain of mice.

c) Each of the different viruses in the mixture could be responsible for a specific and different pathologic manifestation upon injection into newborn mice.

In order to begin to segregate these possibilities, we are studying the nature of the viruses present in the supernatant fluid harvested from our continuous thymocyte cultures. During the past year, we have determined that the supernatant fluid of continuously growing thymocytes derived from the New Zealand Black mice contain not only a murine C-type oncornavirus but also a particulate fraction with a density of $1.19 \text{ gm} \times \text{cm}^{-3}$. This fraction is composed of viral C type and B type (MMTV) particles as well as cellular membranes and organelles. An RNA dependent DNA polymerase (RDDP) as well as a DNA dependent DNA polymerase are present in this fraction, which also contains an endogenous template for the synthesis of DNA. The RNA found in the $1.19 \text{ gm} \times \text{cm}^{-3}$ band does not have a 60-70S sedimentation coefficient which is characteristic of the

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C-type and B-type viruses, but instead sediments at 20-30S. DNA of murine origin is also found in association with the viral core particles which were made by treatment with NP-40. Serial passages of the supernatant fluid from 60A lymphoblast cultures (containing a mixture of C-type virus and particles from the $1.19 \text{ gm} \times \text{cm}^{-3}$ density class) on secondary BALB/c fibroblasts gradually resulted in a loss of the particles with a density of $1.19 \text{ gm} \times \text{cm}^{-3}$, and by the sixth passage none of these particles were observed. Concomitantly, preliminary evidence indicated that the virus progeny obtained from these passages on fibroblasts lost its leukemogenicity, as determined by injection into newborn mice. Because of these results, several questions have become pertinent:

- a) We would like to know what the relationship is between the viral genome and the nucleic acids (DNA and RNA) found in the $1.19 \text{ gm} \times \text{cm}^{-3}$ band.
- b) If the long term animal inoculations corroborate the preliminary evidence that leukemogenicity is lost when the virus is passed on fibroblasts, it will be important for us to determine what correlation can be made between the nucleic acid sequences found in the $1.19 \text{ gm} \times \text{cm}^{-3}$ band and the biological activity of the viruses.

Experiments to answer some of these questions are already in progress. DNA "probes" will be made using the DNA polymerase and template endogenous to either the viral ($1.16 \text{ gm} \times \text{cm}^{-3}$ density class) preparation or to the $1.19 \text{ gm} \times \text{cm}^{-3}$ density class. DNA "probes" will also be made from the virus isolated from the milk of lactating NZB mothers. These "probes" will be hybridized to viral RNA, the RNA and DNA found in the $1.19 \text{ gm} \times \text{cm}^{-3}$ band, to uninfected (whole BALB/c embryo) cell DNA and to DNA extracted from 60A cells. The hybrids will be detected by chromatography on hydroxyapatite and the fraction of the DNA "probe" that hybridizes in the homologous versus the heterologous system will give an indication of the relatedness of the nucleic acids under study.

In order to place all of these studies relative to oncornaviruses and the etiology of autoimmunity into proper perspective, it is necessary to determine which of the many "candidate" viruses are endogenous to one or another colony of New Zealand mice, and if there is any single oncornavirus which seems to be present in all strains of NZ mice. Probably the best way to determine this is to:

- a) determine the genotypic taxonomy of the various viruses in question, and
- b) attempt to isolate in separate laboratories viruses from several colonies of New Zealand mice.

In order to carry out the experiments indicated in a), it is necessary to

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have representative DNA "probes". Experiments in our laboratory as well as in those of Dr. Raymond Gilden and Dr. David Baltimore indicate that the genome of SLV is closely related to that of Moloney virus. These experiments have involved the preparation of DNA "probes" from both viruses using RDDP¹ and the endogenous viral template. Hybridization of RNA extracted from SLV and Moloney to the "probes" was studied. A small difference (2-6%) was observed. None of the laboratories cited have used DNA "probes" with a balanced representation of the viral genome. The "probes" which have been used are total RDDP products, which have been selected by hybridization to a large excess of viral RNA. These "probes" have been shown to be unbalanced with certain viral sequences being represented 10-24 fold more frequently than others. Thus, it is not known whether the small differences observed are the result of lack of representation or correspond truly to a difference between the genomes of the two viruses. We now have the capability for preparation of representative DNA "probes" from the SLV genome by hybridization of the "probe" in DNA excess to radioactively labeled viral RNA. Determination of the extent to which the RNA is protected from digestion by RNase A in high salt is carried out to insure that the RNA is nearly completely resistant to digestion and thus the "probe" is a completely representative complement to the RNA. Digestion of the unhybridized DNA sequences with the endonuclease S₁ or hydroxyapatite chromatography will render a representative DNA copy of the viral genome. If using this DNA "probe" means that the small differences between the genomes of the two viruses still hold true, we will be able to amplify the signal by recycling the unhybridized "probe" and asking the question whether the 2-6% of the SLV "probe" that does not hybridize to Moloney RNA can hybridize 100% to SLV RNA and vice versa. With the recent interest in the xenotropic viruses, a similar experimental approach can be taken to determine the relationship of the xenotropic genome to Moloney and/or SLV viruses. Toward this end, we intend to exchange reagents and "probes" with Dr. Raymond Gilden. To accomplish the objective indicated in b), New Zealand mice have been obtained from Stanford University, Columbia University and we are expecting New Zealand Black mice from Otago University, New Zealand. We will establish lymphoblast cell lines from these mice and those which produce virus will be studied for the relationship of their virus to SLV. In addition, we are studying viral isolates from the NZW and (NZB x NZW)_{F1} hybrid mice. In order to rule out any possibility that we are dealing with a contaminant which originates from a single lab, a collaborative effort will again be undertaken with Dr. Raymond Gilden in which mice are exchanged and lymphoblast lines are established in both laboratories.

6. Possible role of incorporation of exogenous nucleic acids into oncornavirus and other RNA viruses in induction of autoimmunity. One of the basic reasons for suspecting that oncornaviruses may play a role in the etiology of the autoimmunity of the New Zealand mice and perhaps systemic lupus erythematosus in the human is that both these mice and patients with SLE make antibody not only to a single nucleic acid but to both DNA and RNA macromolecules as well as other nuclear and nucleolar macromolecules. Thus, in terms of classical immunology, one was tempted to

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search for an agent which during its natural history exposes the host to as wide a spectrum of potential nucleic acid immunogens as possible. Because of the fact that oncornavirus RNA is transcribed into DNA, and thus a variety of nucleic acid intermediates are involved, they are attractive candidates. Another intriguing possibility is that any virus which might contain both DNA and RNA could be a potential immunogen in autoimmunity. Again, because of the reports of Bishop *et al.*, Smith *et al.* and Puga *et al.* who showed that the avian oncornaviruses, A type particles, and murine oncornaviruses contain DNA as well as RNA, the possibility that oncornaviruses immunize because of fortuitous packaging of both DNA and RNA must be considered. More recently, we have shown that "ordinary" RNA viruses can be made to incorporate cellular DNA. The New Jersey strain of vesicular stomatitis virus, when grown in suspension cultures of diploid human lymphoblasts, packaged host DNA which presumably originates from the plasma membrane associated DNA which we have shown to be present in these cells. This DNA is a linear duplex with a molecular size of approximately 900,000 daltons and by reassociation kinetics can be shown to be of host origin and not to be derived from contamination with viral, mycoplasmic or mitochondrial DNA. Because of the very wide host range, and somatrotrophism of vesicular stomatitis virus, the potential for exchange of genetic information amongst species or different cells within a species is a real possibility. Our initial experiments with the DNA present in the oncornaviruses as well as that present in vesicular stomatitis virus will be aimed at determining the exact relatedness of the DNA in the virion to the host genome. In the oncornavirus system, radioactive DNA precursors will be added to 60A cells and purified $1.19 \text{ gm} \times \text{cm}^{-3}$ particles will be prepared. In order to avoid inherent problems due to possible contamination with random DNA fragments, these particles will be treated with DNase and cores will be made by treatment of the particles with non-ionic detergents and sedimentation either in sucrose or CsCl gradients. Only that DNA which is protected from digestion with DNase and sediments with the ribonucleoprotein cores will be used as a "probe". This DNA will be hybridized to viral RNA, to the RNA and DNA found in the $1.19 \text{ gm} \times \text{cm}^{-3}$ band, and to uninfected (whole BALB/c embryo) cell DNA and to the DNA extracted from 60A cells. By this means, we will be able to determine the origin of the DNA present in the oncornaviruses as well as its relationship to various subfractions of the host genome. It is most likely that this DNA will be similar to that already studied in the avian system and will be representative of the bulk of the murine genome. Similar experiments have already been carried out on the DNA isolated from the cores of vesicular stomatitis virus. Since, as yet, this DNA has not been obtained in large quantities, only limited studies relative to the analytical complexity of this DNA have been performed. Nevertheless, these studies are of extreme interest, in that they indicate that only unique sequences of the lymphocyte genome are packaged into the VSV cores. During the next year, we intend to study in great detail the analytical complexity of the DNA contained in the vesicular stomatitis virus cores to determine its exact relationship to the human genome. One intriguing aspect of all this is that there is a rearrangement of sequence relatedness and complexity relative to the host genome. For example, even though a sequence may be repeated many, many times in the host genome, if only one copy is present in the virions derived from those cells, then as far as

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the virion mass is concerned, this sequence is unique. Once we determine the analytical complexity of the DNA present in the VSV cores, we intend to turn to the question of whether vesicular stomatitis virus can pick up "oncogenes" and deliver them in a meaningful fashion to cells from different species and/or different somatic types. In many respects, VSV is an ideal virus for such studies. As mentioned above, it has a wide host range and somatotrophism. Perhaps more important, because it contains a large number of defective interfering particles which do not kill host cells, the potential for a meaningful exchange of genetic information is enhanced. We plan to infect with VSV malignant cells which are not producing RNA or infectious virus. After infection with VSV, the T or defective interfering particles will be extensively purified and their oncogenic potential will be studied in both in vitro and in vivo systems. If these particles can be shown to be oncogenic, then a very important system for encapsulation of "oncogenes" may be realized. In addition, with respect to their autoimmunogenic potential, we will immunize animals with similar particles and determine whether they can induce autoimmunity in susceptible strains. Again, one reason to suspect that this may work is that such particles may induce cells to replicate and synthesize nucleic acids foreign to the host even in the absence of production of complete virus.

7. Relationship of virion coded for and/or induced phenotypic markers to oncogenesis and autoimmunity.

Cells transformed by oncornaviruses are characterized by a number of "markers" such as: 1) production of complete virus particles (electron microscopy), 2) production of infectious virus particles (XC), 3) presence of RNA dependent DNA polymerase, 4) presence of GP70 on the cell surface (interspec 2, OSA), 5) presence of P30 (gs-1), 6) oncogenicity.

Continuous lymphoblast cultures from NZB mice (SCRF 60A) have all of these "markers". To determine the relationship between each of these markers and the transformed phenotypes, SCRF 60A will be cloned in microculture plates. Replicate cultures can then be obtained using a replicate plating device designed by Dr. Jim Robb. The master culture can then be frozen using dimethylsulfoxide until variant cells can be characterized. With antisera prepared in our lab, we can then select by cytotoxicity and screen by immunofluorescence literally thousands of clones to obtain cells with phenotypes such as OSA^{gs+}, OSA+XC⁻, etc. Certain variant cells can then be grown to mass culture and tested for their oncogenic potential in syngeneic adult and newborn mice. These variant cells will allow us to determine which of the virus coded and/or induced phenotypes are necessary for oncogenesis and which, if any, confer negative selective pressure for tumor growth. For example, from the data which we already have from our study of spontaneously occurring solid tumors, one might expect OSA^{gs+} or OSA-rt⁺ cells to be highly oncogenic.

The virus produced by SCRF 60A cells (SLV 60A) has been shown to be a mixture of particles, some of which have a defective genome and/or contain cellular DNA. SLV 60A can be used to infect mouse or rat embryo fibroblasts in microculture plates. The techniques described above can then be applied to these cultures to clone viruses with defective genomes. In fact, this technique

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could be used to isolate temperature sensitive mutants of SLV. By this approach, we may be able to further elucidate the role of the various viral functions in oncogenesis and autoimmunity. To establish this end, cellular variants or defective viruses will be injected into newborn mice and the mice studied for the development of tumors and autoimmune disease. As far as autoimmunity is concerned, it will be interesting if the cell which has a phenotype such as V^- , OSA^- , rt^+ , gs^- could, because of production of a potential array of nucleic acid immunogens, induce autoimmunity. It, of course, remains to be determined if cells or viruses with the exact phenotypes which we would like to have can be obtained, but this seems to us to be an important approach to the problem of the relationship between autoimmunity and neoplasia.

In addition, DNA-DNA reassociation kinetics will be utilized to compare the number and completeness of SLV genome equivalents present in the variant cell genome to that of virus infected cells so as to determine whether a complete gene complement is present. In this way, we should be able to discriminate between the absence of a complete genome and the failure to express certain genes. To do this we will utilize DNA "probes" which were prepared last year. In the preparation of these "probes", we were careful to select DNA molecules which are most representative of the complete viral genome. We expect this work to complement our ongoing effort which has already shown that, at least as far as the New Zealand mice are concerned, the spontaneously occurring solid tumors rarely, if ever, express all the markers indicated above.

8. Search for C-type virus in humans .

Recently it has been shown that C-type viruses are present in the syncytial trophoblasts of primates and humans. Because of the possible role of these viruses in the etiology and pathogenesis of systemic lupus erythematosus (SLE), we are making attempts to isolate and characterize a C-type virus from the placentas of patients with SLE. To date we have obtained four placentas from SLE patients -- two preterm and two term. For controls we have obtained five normal, three preterm and two term placentas.

Part of the placentas were fixed for histologic and electron microscopic studies, part were used for establishment of tissue cultures and the remainder frozen for future studies.

For positive control murine placentas from high leukemic strain AKR and low leukemic strain NIH Swiss were used.

In the preterm SLE placenta (so far the only SLE placenta examined by electron microscopy) C-type virus buds and particles were found in the lateral intercellular spaces of cytotrophoblasts. The virus particles are present in various stages of budding. The complete particles are 90-100 nm in diameter and have the typical appearance of C-type virus as seen in AKR or NIH Swiss mice. The relative frequency of particles and buds per area of EM thin section are given below:

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Human SLE placenta	$5.5 \times 10^2/\text{mm}^2$
Human Control placenta	$<1/\text{mm}^2$
Murine NIH Swiss placenta	$1 \times 10^2/\text{mm}^2$
Murine AKR/J placenta	$20 \times 10^2/\text{mm}^2$

C. Virological studies. Co-cultivations of SLE trophoblasts with human embryonic fibroblasts, human lymphoblasts, primate fibroblasts, canine embryonic fibroblasts and rat fibroblasts are in progress. Supernatant fluids from these cultures will be assayed for the presence of an RNA dependent DNA polymerase in association with an endogenous template. DNA probes will be made from the RDDP positive supernatants. They will be hybridized to RNA extracted from murine, feline and primate C-type viruses, as well as to the endogenous RNA that served as template for the synthesis of the DNA probe. If these hybridizations show that the DNA sequences are specific to the RNA on which they were synthesized and do not hybridize to viral RNA from other mammalian C-type viruses, their relationship to human DNA sequences and their evolutionary relationship to other primate DNA will be studied.

A quantitative infectivity assay system similar to the XC test will be developed. We have acquired the personnel and methodology to characterize virus isolates and study virion proteins by biochemical and immunological means.

We have excellent capabilities in (1) production of large amounts of cells and virus, (2) iodination of cell surfaces and virion proteins and (3) determination of their group, type and species specificity by radioimmunoassay.

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11. Publications or papers in press resulting from this work

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12. Summary Progress Report

and nucleolar macromolecules during the cell cycle.

In the past year we have made considerable progress toward all of the objectives listed in our proposal last year. The report of our activities which follows is listed under the following headings.

1. Effects of neonatal infection of mice and rats with SLV.
2. Immunization of NZB x W female mice with formalinized SLV vaccine.
3. Isolation and characterization of an oncornavirus surface glycoprotein.
4. Taxonomy of Scripps leukemia virus (SLV).
5. Somatotrophic variation of the molecular properties of oncornaviruses.
6. Lack of viral expression in spontaneous tumors of NZB x W F_1 and NZB mice.
7. Biophysical properties of plasma membrane associated DNA.
8. Immunoelectron microscopic study of the structure of nucleic acids.
9. Quantitation and isolation of plasma membrane associated immunoglobulin.
10. Fusion of lymphocytes by feline leukemia virus.
11. Search for C-type viruses in humans.

1. Effects of Neonatal Infection with SLV

In order to determine the oncogenic and autoimmunogenic potential of SLV, the oncornavirus isolated from NZB lymphoblasts (3), we have inoculated SLV into neonatal mice of 16 strains including NZB, NZW and NZB x W. The strains were selected to include both B and N types and a variety of H-2 types so that the influence of these genetic factors in the propagation of virus and causation of disease might be observed.

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THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

January 7, 1974

Grant Application No. 951

CANCER

To: The committee comprising Drs. ^{Gardner} Andervont, Huebner and Jacobson

Subject: Hans Meier, D.V.M., The Jackson Laboratory, Bar Harbor
New application No. 951
"Transplacental effects of Nitrosocompounds in Inbred
Strains of Mice and Rabbits"

History

In a letter dated 18 December 1973 Dr. Meier indicates that the enclosed proposal is, at least in part, an outgrowth of his ongoing study "Oncogenesis in the rabbit . . ." supported by CTR grant 758BR1 (Dr. Meier is currently requesting a renewal of this grant).

Request

Application No. 951 requests \$25,461 plus two additional years.

Document Submitted

Attached is an application dated 1 January 1974, including CV's of the three investigators (16 pages).

Comment

We may seek evaluation of Dr. Meier's new proposal by an outside consultant.

FWN:gh

Encls.

F.W.N.
F.W.N.

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#951

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022

Application For Research Grant

RECEIVED
(DEC 26 1973)

Date: 1 January 1974

1. Name of Investigator(s): (include Title and Degrees)

Hans Meier, D.V.M., Dr. med. vet., M.R.S.H., Senior Staff Scientist
R. R. Fox, Ph.D., Staff Scientist, and B. A. Diwan, Ph.D., Research Associate

2. Institution &

Address: The Jackson Laboratory, Bar Harbor, Maine 04609

3. Short Title of Project: Transplacental effects of nitrosocompounds in inbred strains of mice and rabbits.

4. Proposed Starting Date: 1 July 1974

5. Anticipated Duration of this Specific Study: 3 years

6. Brief Description of Objectives or Specific Aims: Attached

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7. Give a Brief Statement of your Working Hypothesis: Attached

Details of Experimental Design and Procedures: (Attach Separate Pages)

Attached

Physical Facilities Available (Where Other than Administering Organization Indicate Geographical Location)

The Jackson Laboratory, Main Complex, and Hamilton Laboratory, West

Additional Requirements: None

Biographical sketches of all principal and professional personnel (append)

Attached

List of publications: (Five most recent as pertinent) (append)

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13. Budget: (1st year)

A. Salaries (Personnel by names)

Professional

Hans Meier, Project Director
R. R. Fox, Co-Project Director
B. A. Diwan, Research Associate

% time

5
5
50

Amount

REDACTED

Technical

R. F. Norberg, Research Assistant
Eugene Farrin, Research Assistant
Animal Caretaking
Secretarial Assistance

15
20

REDACTED

Sub-Total

REDACTED

Employee benefits: 16%

B. Consumable Supplies (list by categories)

Chemicals, glassware, instruments
Food and Bedding

1,000
900

Sub-Total

1,900

C. Other Expenses (itemize)

Domestic travel to scientific meetings
Publication Costs
Art, Photo, and Replicating Service
Histotechnical Service
Mouse Purchases

250
200
200
1,000
1,400

Sub-Total

3,050

D. Permanent Equipment (itemize)

None

E. Overhead (15% of A + B + C)

3,321
Total \$25,461

Estimated Future Requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Overhead	Total
Year 2	A	\$2,000	\$3,275	-	\$3,523	\$27,010
Year 3	B	\$2,100	\$3,500	-	\$3,728	\$28,584

It is understood that the applicant and institutional officers
in applying for a grant have read and found acceptable
the Council's "Statement of Policy Containing Conditions
and Terms Under Which Project Grants Are Made."

Signature

Director of Project

Signature

Business Officer of the Institution

Telephone

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Other Sources of Financial Support

List financial support for research from all sources, including own institution, for this and/or related research projects.

Current	Title of Project	Source	Amount	Duration
	mcogenesis in the rabbit; genetic susceptibility, vertical transmission of virus, and environmental influences.	The Council for Tobacco Research, U.S.A.	\$ 23,310	6/1/73-5/31/74
	Natural occurrence of RNA tumor virus (genomes) and host-gene control of their expressions.	National Cancer Institute	400,000	5/1/73-4/30/74
	Hereditary diseases and variations of the rabbit.	NIH General Research Support grant to the Jackson Laboratory	41,731*	7/1/73-12/31/73
	Rabbit inbred and mutant stocks resource.	NIH Division of Research Resources	25,000*	1/1/73-12/31/73
ending	mcogenesis in the rabbit; genetic susceptibility, vertical transmission of virus, and environmental influences.	The Council for Tobacco Research, U.S.A.	24,401	6/1/74-5/31/75
	Hereditary diseases.	National Institute of General Medical Sciences	35,315*	5/1/74-4/30/75
	Rabbit inbred and mutant stocks resource.	NIH Division of Research Resources	98,015*	1/1/74-12/31/74
			*Direct costs	

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6. Objectives and specific aims

The objectives of this investigation are to analyse the transplacental embryotoxic, teratogenic, and carcinogenic effects of two classes of nitrosocompounds, nitrosoureas and nitrosamines, in inbred strains of mice and rabbits. Obviously, these studies are complex, and some narrowing of aims is necessary. Thus, we propose to evaluate the action of the compounds with respect to (1) their retention, activation (conversion), and degradation, (2) the role of fetal age or gestational days, and (3) the mode of inheritance and number of genetic loci involved in species and strain differences. We feel that detailed definition of these goals is pertinent to the situation in man: (a) malformations, congenital tumors, and childhood cancers may have been transplacentally induced, and (b) at least certain types of tumors in adults may be due in part to delayed consequences of carcinogen-exposure during intrauterine life.

Strains of mice chosen for study are: AKR/J, DBA/2J, SWR/J, C57BL/6J, and C57L/J; they differ from one another by genetic origin (except for the last two), type C RNA tumor virus expression, inducibility of aryl hydrocarbon hydroxylase (AHH), and sensitivity to 1-ethyl-1-nitrosourea (ENU)-induced teratogenicity and carcinogenicity among other traits. For studies with rabbits we are primarily focusing on strains III and WH because of their differences in genetic origin and AHH inducibility.

7. Working hypothesis

Transplacental exposure is an extremely sensitive method for study of the biological effects of potentially harmful pollutants. Their effects depend upon hereditarily determined differences in susceptibility or resistance of pregnant mothers and embryos, and the stage of fetal (organ) development.

8. Experimental design and procedures

1. Introduction. The potential hazard to man and animals of environmental pollutants is becoming increasingly apparent both upon acute and chronic exposure. Since developing fetuses are particularly susceptible, malformations, congenital and childhood cancers, as well as tumors in adults, may in part be due to consequences of intrauterine chemical injury. Transplacental embryotoxic, teratogenic, or carcinogenic effects have been demonstrated experimentally for numerous compounds particularly polycyclic hydrocarbons and nitrosocompounds. Differences in their effects and causes have been conjectured as due to consequences of species, gestational stage, dosage, and mode of action. Very few studies concern detailed investigations of multiple variables including genetic analyses. Because of the widespread occurrence of nitrosocompounds in industrial pollution, foods, e.g. cured meat, smoked fish, etc., and tobacco products, we wish to gain a better understanding of their specific biological effects.

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The discovery of the toxic effects and carcinogenicity of N-nitrosocompounds was purely accidental. For example, dimethylnitrosamine (DMNA) had been incriminated as the cause of a high incidence of liver cirrhosis among workers of an English factory (1). Aside from and in addition to toxic effects, administration of DMNA to rodents readily produced hepatic carcinomas (2). Depending upon the dose, route, and type of compound an entire spectrum of tumors has been induced in different species including neurogenic tumors with urea derivatives or acyl-alkyl nitrosamides (3). Smoke condensates contain up to nine different nitrosamines depending upon the

type of cigarette, and their level(s) is related to those of total volatile bases and the tobacco nitrate content (4).

Our aims will focus initially on acute studies, using single transplacental administration of the various N-nitrosocompounds. They will then be extended to chronic low-level exposures during the entire duration of pregnancy, including pretreatments of prospective mothers.

2. Details of Experimental Design and Procedure. There are two classes of nitrosocompounds with respect to their mode of action, (a) nitrosoureas that are direct-acting, or proximate carcinogens and (b) nitrosamines that require metabolic modification. We wish to study the transplacental effects of the two nitrosamines, dimethyl- and diethylnitrosamine (DMNA and DENA) relative to 1-ethyl-1-nitrosourea (ENU). Whereas metabolic activation of DMNA and DENA apparently involves oxidative dealkylation, the outcome of ENU-induced effects most likely depends on genetically determined differences in the cellular repair mechanism rather than its metabolism or transplacental uptake. Since enzyme synthesis is generally repressed in fetal tissues, but related to the stage of differentiation, the carcinogenic effects of DMNA and DENA are probably more rigorously stage- and strain-dependent than those of ENU. Thus, the relationship of fetal enzymatic competence and the genetically dependent expression of endogenous type C-RNA tumor virus may be more precisely identified than in case of ENU. Also, certain of the strains used for these studies are aryl hydrocarbon hydroxylase (AHH)-inducible (C57L/J, C57BL/6J, SWR/J, SWR/J), whereas others (DBA/2J, AKR/J) are not. Apparently high levels of AHH depress DMNA-demethylase.

Studies in inbred strains of mice

1-ethyl-1-nitrosourea. Single intraperitoneal injections of 0.5 mmoles/kg of 1-ethyl-1-nitrosourea (ENU) were given to mice of strains C57L/J, C57BL/6J, SWR/J, DBA/2J, and AKR/J on days 8 and 12 of gestation, and fetuses examined on day 14 or after birth. Both the frequency and severity of ENU-induced malformations varied with the stage of embryogenesis and the strain of mice; the strains of mice are listed in decreasing order of susceptibility (5). Tumors were produced upon treatment of the same strains on days 14, 16, and 18 of gestation. Again, the incidence, types, and latency periods of the induced tumors were strain- and age-dependent, but the susceptibility to transplacental ENU-carcinogenesis was inverse to that for ENU-teratogenesis (6). Assays for the expression of type C RNA tumor virus or its group-specific antigen (gs-AG) in tissues of different strains of mice indicated that (a) viral activation occurred in mice after transplacental administration of ENU, (b) a significant association existed between gs-AG and in some cases complete virus expression and ENU-induced tumors, and (c) the rate of tumor induction and gs-AG or virus expression was influenced by the host genotype or strain of mice (7). Reciprocal F₁ hybrids of strains AKR/J and SWR/J were exposed to transplacental treatment of ENU on day 16 of gestation. A pronounced maternal influence on the development of tumors was observed. Thus (SWR/J ♀ x SWR/J ♂)F₁ offspring were found to be more susceptible to the induction of lung tumors and leukemia (latency 10 weeks or less) by ENU than the offspring of (AKR/J ♀ x SWR/J ♂)F₁ mice (16 to 20 weeks). Because of the consistently high incidence of development of multiple (two or more types of) tumors within a short period of time, (SWR/J ♀ x AKR/J ♂)F₁ mice provide a highly sensitive model for the simultaneous testing of anti-type C RNA tumor viral vaccines against both mesenchymal and epithelial tumors (8).

Chronic low dose ENU exposure of mice during the entire course of pregnancy

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and of prospective mothers has not yet been investigated.
and the tobacco nitrosamine content (4).

Dimethyl- and diethylnitrosamine. The importance of fetal metabolizing enzymes relative to the effects of DMNA and DENA may best be studied upon administration of these compounds at various gestational days, i.e. 12, 14, 16, and 18 days. Apparently, their role is reflected in the formation of alkylating intermediates following dealkylation by microsomal mixed oxidase enzymes. According to Preussmann et al. (9), the amount of formaldehyde liberated from fetal liver- and kidney-microsomes can be used to determine the activity of DMNA-demethylase. We plan to utilize a modification of the demethylase assay of Ventkatsan et al. (10) and determine the extent of formaldehyde-liberation from isolated microsomes following the procedure of Chochin and Axelrod (11); protein will be estimated by a microbiuret method (12).

DENA-dealkylation will be determined in fetal livers and kidneys using procedures described by Preussmann et al. (9) and Stotz (13); this method is based upon a color-reaction of acetaldehyde with a copper sulfate-p-hydroxy-biphenyl reagent.

Embryotoxicity, teratogenicity, and carcinogenicity assays will be performed in treated versus control (vehicle-injected) mice. Standard genetic analyses will be conducted for possible correlations between dealkylating activity by organ, gestational days, and organ-specific tumorigenesis.

Studies in rabbits

1-ethyl-1-nitrosourea. Our preliminary results on the effects of ENU in strain-crosses segregating for alleles at the Ah locus tend to indicate a possible association with AHH inducibility, despite the fact that the fetal genotype appears irrelevant. If strain differences in dealkylation, tumor induction, and virus-expression are observed, genetic analyses may provide us with answers to their mode of inheritance and the number of genes involved.

Strain III_{vo} and III_{mo} fetuses are highly susceptible to ENU-induced renal tumors when pregnant mothers are treated on day 18 of pregnancy; multiple tubular adenomas and adenocarcinomas develop as early as 40 days after birth. These tumors occur reproducibly and within a limited time, and provide us with an opportunity to search for endogenous rabbit C-type tumor virus(es) as outlined in CTR-RENEWAL APPLICATION 758. Pregnant rabbits of two partially inbred strains III and WH were injected intraperitoneally with a single dose of 60 mg/kg of ENU in trioctanoin on day 18 of gestation. Controls were treated on the same day with solvent alone. Tumors developed in almost all offspring of strain III that survived more than 8 weeks. In contrast, no such tumors have yet developed in offspring of strain WH (14). Renal tumors developed in offspring of strain III rabbits at an exceptionally high incidence (70%). Induction time ranged from 40 days to 135 days. Kidney tumors, either renal adenomas or adenocarcinomas appeared to develop within cysts occurring in subline III_{vo} because of homozygosity for a recessive gene causing renal cyst formation (rc).

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Dimethyl- and diethylnitrosamine. The procedural details are analogous to those described for mice. With respect to retention, conversion, and degradation of N-nitrosocompounds, a number of radiolabels for their radicals are available for analysis. Also, the nitroso-tumor models are highly suitable for testing the "oncogene" hypothesis (see RENEWAL APPLICATION 758) since virus-particles (type C?) have been observed on a few occasions even in neurogenic tumors, although their significance in the pathogenesis of experimental tumors is as yet unknown.

Methylcholanthrene (MCA) induced amino azo N-demethylase and represses DMNA-N-demethylase synthesis in rat livers (15). These findings were used to explain the fact that hepatocarcinogenesis was not enhanced by simultaneous treatment with MCA and DMNA (16). However, synergetic effect was found with respect to lung tumorigenesis in rats; also the combined application of MCA and DMNA in mice lead to a higher overall tumor incidence than found by either DMNA or MCA application alone (17). Thus, remarkable species and organ differences were found in rats and mice. In order to resolve this situation, it is essential to study this system in other species of laboratory animals. We, therefore, plan to investigate the combined effects of MCA and DMNA in rabbits with respect to their possible syncarcinogenicity, using AHH-inducible strain III and AHH-noninducible strain WH.

3. Significance of this research. Products necessary for the synthesis of nitrosocompounds occur in the environment (18, 19). For example, nitrosamines are readily produced upon reaction of nitrous acid or sodium nitrite on secondary amines or alkylamides. Nitrite- and nitrate-yielding substances occur in a variety of drugs and food additives. Secondary amines and alkylamides are also widespread, e.g., in bacteria, fish, etc., (20, 21), and are produced during cooking processes. A number of them are used as industrial solvents and neutralizing agents. Nitrosamines have also been detected in tobacco smoke, especially in smoke from cigarettes containing a high content of nitrate (4, 22). Apparently nitrogen oxide (nitric oxide and nitric dioxide) and volatile bases of tobacco smoke are the precursors of N-nitrosamines. Oxides of nitrogen may react with secondary amines, particularly nor nicotine and anabasine of smoke to form nitrosoanabasine and nitroso nor nicotine (23).

Our current studies in rabbits with 1-ethyl-1-nitrosourea show that primary renal tumors, i.e., papillary cyst-adenomas and mixed Wilms'-like nephroblastomas, can readily be induced in strain III rabbits with a single dose of ENU given on day 18 of pregnancy. Apparently, the incidence and inducibility depend upon the presence or absence of homozygosity for a gene, rc, responsible for renal cortical cyst formation (24). The occurrence and distribution of this gene is high in subline III_{vo} and low in subline III_{mo}; it is absent in strain WH. The pathogenic significance of renal cyst formation to primary renal tumor induction is indicated by both the origin of the tumors from cyst epithelium and their multicentricity. Thus, whereas genotypic differences are of obvious importance to renal tumorigenesis, susceptible strain III_{vo} rabbits develop renal tumors even when given a single intraperitoneal injection of ENU at 8 weeks of age (unpublished results). We are now analysing the effects of other classes of carcinogens including different nitrosocompounds on the susceptibility or resistance to renal tumor development in strain III rabbits. Also, genetic analyses of renal tumor susceptibility are in progress utilizing all possible crosses between strains III and WH.

It is noteworthy that we have found a single case each of nephroblastoma or Wilms' tumor in old WH and strain III rabbits (25); yet, strain WH is entirely resistant to the transplacental induction of renal tumors, whereas strain III appears to be highly susceptible. The only other case of spontaneous Wilms' tumor occurred in a strain AC rabbit. In contrast, none were ever found in strains OS, ACEP, AX, A, C, III_c, and hybrids between III and III_c (25). Thus, the occurrence of spontaneous and induced primary renal tumors, including Wilms'-like tumors in rabbits is genotype-dependent. Although the frequency of Wilms' tumors is low in man, the occurrence in clusters among sibships, families, successive generations, and identical twins also suggest an hereditary tendency (26-32). A critical relationship between tumor-induction or occurrence and renal cyst formation could exist in man as well as the rabbit because polycystic renal disease of man is known

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genesis with 1-aryl-3,3 dialkyltriazenes. Enzymatic dealkylation by rat liver microsomal fraction in vitro. *Biochem. Pharmacol.* 18:1-13.

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11. COCHIN, J., and W. A. E. KARUNARTNE. 1936. Carbon tetrachloride cirrhosis in relation to liver regeneration. *J. Path. Bact.* 42:1-21.

12. GORNALL, A. G., C. J. BARDAWILL, and M. M. DAVID. 1949. Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.* 177:741-766.

13. STOTZ, E. 1963. A colorimetric determination of acetaldehyde in blood. *J. Biol. Chem.* 148:585-591.

14. FOX, R. R., B. A. DIWAN, and H. MEIER. Induction of primary renal tumors in rabbits treated with ethylnitrosourea. *J. Nat. Cancer Inst. (under preparation)*.

15. ARGUS, M. F., R. T. VALLE, N. VENKATESAN, N. P. BUU-HOI, and J. C. ARCOS. Molecular-size-dependent effects of polynuclear hydrocarbons on mixed function oxidases: Possible action on cascade coupled operons. *Separatum, First european biophysics congress, Sept. 1971, Edition Wiener Med. Akademie, pp. 187-192.*

16. HOCH-LIGETI, C., M. F. ARGUS, and J. C. ARCOS. 1959. Combined carcinogenic effects of dimethylnitrosamine and 3-methylcholanthrene in the rat. *J. Nat. Cancer Inst.* 40:535-549.

17. CARDESA, A., P. POUR, M. RUSTIA, J. ALTHOFF, and U. MOHR. 1973. The syncarcinogenic effect of methylcholanthrene and dimethylnitrosamine in Swiss mice. *Z. Krebsforsch.* 79:98-107.

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19. SANDER, J., and F. SCHWEINSBERG. 1972. Wechselwirkungen zwischen Nitrate, Nitrite und kanzerogenen N-nitrosoverbindungen. *Zentralbl. f. Bakt., Parasitenk., Infektionskrankh., und Hygiene (in press)*.

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22. RHOADES, J. W., and D. E. JOHNSON. 1972. N-dimethylnitrosamine in tobacco smoke. *Nature* 236:307-308.

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25. FOX, R. R., H. MEIER, and D. D. CRARY. 1971. Genetic predisposition to tumors in the rabbit. *Naturwissenschaften* 58:457-458.
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33. DALGARD, O. Z. 1957. Bilateral polycystic disease of the kidneys--a follow-up of 284 patients and their families. *Acta Medica Scandinavica* (Suppl. 328) 158:1-252.

Biographical sketches of all principal and professional personnel

Hans Meier

Born:

Nationality:

Education:

University of Zurich (Veterinary Medicine), D.V.M.

University of Zurich (Pathology)
Dr. med. vet.

Harvard School of Public Health

Massachusetts Institute of Technology, Alumnus (Biophysical and biochemical cytology, biology)

Honors:

Royal Society of Health
(Pharmacology), M.R.S.H.

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1963

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Member, Scientific Advisory Board,
The Council for Tobacco Research, USA 1971-

Member, Solid Tumor-Virus Working
Group, Special Virus-Cancer Program,
National Cancer Institute, NIH 1971-1973

Member, Breast Cancer Virus Segment,
National Cancer Institute, NIH 1973-

Major Interests:

Experimental pathology, cancer

Positions:

1970- The Jackson Laboratory, Senior
Staff Scientist

1970- The National Cancer Institute,
Consultant

1962-70 The Jackson Laboratory, Staff
Scientist

1960-62 The Jackson Laboratory, Associate
Staff Scientist

1957-60 Harvard Medical School, Res. Asst.
(Pathology)

1958-60 Children's Hospital and Medical
Center, Boston, Asst. (Experi-
mental) Pathologist

1957-60 Children's Cancer Research Foundation
Res. Asst., Res. Assoc. (Exp. Pathol.)

1954-57 Angell Memorial Hospital, Boston,
Fellow, Resident, Chief of Pathology

Postgraduate
training:

1955-57 Harvard Medical School, Course work
in pathology and neuro-pathology

1957 Harvard University, Summer School of
Arts and Sciences, and of Education
(Biophysics and radioactive measure-
ments)

1958 Armed Forces Institute of Pathology
(Course in histochemistry)

1959 Harvard University, Depts. Biology,
(Electron microscopy) and Chemistry
(Experimental methods in biochem.)

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Societies:

Richard R. Fox

Born:

Nationality:

Educated:

University of Connecticut (Animal Production)
B.S.

University of Minnesota (Animal Breeding)
M.S.

University of Minnesota (Animal Breeding
and Genetics) Ph.D.

The Jackson Laboratory (Rabbit Genetics),
Postdoctoral Fellow

Honors:

B.S., with honors and with distinction in
Animal Production

Sigma Xi

Gamma Sigma Delta

Alpha Zeta

Member: Committee on Nomenclature for Inbred
Rats, Hamsters, Gerbils, and Rabbits, Nat.
Acad. Sci., ILAR

Major interests:

Genetics of the rabbit; quantitative genetics;
reproductive physiology; animal husbandry

Positions:

1957-1965 The Jackson Laboratory, Staff
Scientist

1961- University of Maine, Department
of Animal and Veterinary Science,
Lecturer

1960-65 The Jackson Laboratory, Assoc.
Staff Scientist

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Societies:

REDACTED

Bhalchandra Apparo Diwan

Born:

REDACTED

Nationality:

REDACTED

Education:

Willington College, Sangli,
B.Sc. (Gen.)

REDACTED

1956

University of Poona

B.Sc. (Hons.)

M.Sc.

Ph.D.

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Academic honors:

Recipient of an award for highest
marks in chemistry, Willington
College, Sangli, India

1959

Research scholar of Govt. of
India, Ministry of Education,
New Delhi, India

1961-1966

Major interests:

Viral-chemical cocarcinogenesis,
embryology

Positions:

1971- The Jackson Laboratory
Postdoctoral Trainee1967-1971 Cancer Research Institute,
Parel, Bombay, Scientific
Officer1966-1967 Vivekanand College, Kolhapur
Professor of Zoology1964-1966 Indian Council of Medical Research,
New Delhi, India
Postdoctoral Fellow

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Relevant publications

MEIER, H., D. D. MYERS, and R. J. HUEBNER. 1969. Genetic control by the hr-locus of susceptibility and resistance to leukemia. Proc. Nat. Acad. Sci., USA 63:759-766.

FOX, R. R., H. MEIER, DORCAS D. CRARY, D. D. MYERS, R. F. NORBERG, and C. W. LAIRD. 1970. Lymphosarcoma in the rabbit: genetics and pathology. J. Nat. Cancer Inst. 45:719-729. (Supported in part by CTR).

HUEBNER, R. J., G. J. TODARO, P. SARMA, J. W. HARTLEY, A. E. FREEMAN, R. L. PETERS, C. E. WHITMIRE, and H. MEIER. 1970. "Switched-off" vertically transmitted C-type RNA tumor viruses as determinants of spontaneous and induced cancer: a new hypothesis of viral carcinogenesis. Proc. 2nd Int. Symp. on Tumor Viruses, Paris, France, No. 183:33-57.

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MEIER, H., R. L. HANCOCK, and R. J. HUEBNER. 1970. Effect of poly rI: rC on sRNA methylase in leukemic and normal spleens. Life Sci. 9:641-651.

MEIER, H., D. D. MYERS, R. R. FOX, and C. W. LAIRD. 1970. Occurrence, pathological features, and propagation of gonadal teratomas in inbred mice and the rabbit. Cancer Res. 30:30-34.

MEIER, H., D. D. MYERS, and R. J. HUEBNER. 1970. Differential effect of a synthetic poly-ribonucleotide complex on spontaneous and transplanted leukemia in mice. Life Sci. 9:653-659.

MYERS, D. D., H. MEIER, and R. J. HUEBNER. 1970. Prevalence of C-type RNA virus group-specific antigen in inbred strains of mice. Life Sci. 9:1071-1080.

FOX, R. R., H. MEIER, DORCAS D. CRARY, R. F. NORBERG, and D. D. MYERS. 1971. Hemolytic anemia associated with thymoma in the rabbit: genetic studies and pathological findings. Oncology 25:372-382. (Supported in part by CTR).

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MEIER, H. and R. J. HUEBNER. 1971. Host-gene control of C-type RNA tumor virus expressions: relevance of studies in inbred mice to cancer in man and other species. Proc. Nat. Acad. Sci., USA 68:2664-2668.

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Budget Justification

There is no need to include in this budget more than a small salary provision for the efforts contributed by the Project Directors, because this proposal relates to work supported by NIH research contract N01 CP 33255 from the National Cancer Institute and research grant #758 from the Council for Tobacco Research--USA.

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#973-RASMUSSEN

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THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

- February 7, 1974

Grant application No. 973

CANCER

To: The committee comprising Drs. Gardner, Huebner,
Meier

Subject: Ronald E. Rasmussen, Ph.D., University of Calif.
New application No. 973
"Effect of Cocarcinogens and Tumor Promoters on DNA
Repair in Mammalian Cells Susceptible to Chemical
Transformation"

History

A preliminary inquiry was handled as Case No. 149 in
the Fall of 1972. The action was to defer consideration until
towards the end of 1973.

Request

Application No. 973 requests \$34,645, plus one addi-
tional year.

Documents Submitted (attached)

1. Application dated 1-30-74 (5 pages plus inserts).
2. Curriculum Vitae and bibliography of Dr.
Rasmussen.

(We have left stapled to the application, as submitted,
a number of reprints.)

FWN:wg
Encls.

FWN
F.W.N.

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4973

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

FEB 4 1974

Application for Research Grant
(Use extra pages as needed)

Date: 1-30-74

1. Principal Investigator (give title and degrees):

Ronald E. Rasmussen, Ph.D. , Assistant Research Physiologist, Cancer Research Institute.

2. Institution & address:

University of California, 3rd Ave. at Parnassus, San Francisco, CA., 94143.

3. Department(s) where research will be done or collaboration provided:

Cancer Research Institute

4. Short title of study:

Effect of Cocarcinogens and Tumor Promoters on DNA Repair in Mammalian Cells Susceptible to Chemical Transformation.

5. Proposed starting date: April 1, 1974

6. Estimated time to complete: 2 years

7. Brief description of specific research aims:

Cell cultures derived from NIH-Swiss mouse embryos will be given a sublethal dose of ultraviolet (UV) light which will produce a known amount of DNA damage and result in a readily measurable amount of DNA repair. Before, after, or simultaneously with the UV exposure, the cells will also be exposed to specific carcinogens, cocarcinogens, or tumor promoters, and the amount of DNA repair (as indicated by incorporation of radioactive precursors into DNA under the conditions described below) will be compared to control cultures not so exposed. The results will bear on the question of whether the compounds tested exert their effects through interfering with DNA repair, and which compounds may be particularly important in this regard. Concurrently, the levels of hydroxylating enzymes (ANH) in the cell cultures and the toxic effects of the test compounds in terms of inhibition of cell proliferation will be measured.

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8. Brief statement of working hypothesis:

2.

DNA is the repository of genetic information in mammalian cells and, as such, its integrity must be maintained. This is thought to be accomplished, in part, by a DNA repair system that is capable of recognizing damaged or distorted regions of DNA, excising them, and restoring the removed region with the proper sequence of nucleotides. It is known that many carcinogenic chemicals bind to DNA both in vivo and in vitro, and further that often this binding stimulates the DNA repair system. Evidence that DNA repair may be closely related to human cancer has been provided by studies of the hereditary disease xeroderma pigmentosum, which is characterized by extreme sensitivity to UV light (e.g. sunlight) and the subsequent development of skin cancer. The DNA repair system has been shown to be defective in individuals with this disease.

Given the relationship among DNA damage, DNA repair, and carcinogenesis suggested by the above findings, it is of interest to determine whether cocarcinogens and tumor promoters may act by interfering with DNA repair. The results of these studies will provide basic information regarding the action of carcinogenic substances, and may indicate the mode of action of the modifiers of carcinogenic activity.

9. Details of experimental design and procedures (append extra pages as necessary)

INTRODUCTION AND BACKGROUND

Repair of Damage to DNA. Damage to the genome (DNA) of mammalian cells or bacteria, whether caused by radiation or certain chemicals, results in the initiation of a special kind of DNA synthesis that has been equated with the repair of the lesion. This process has been called "uncheduled DNA synthesis", "radiation-stimulated DNA synthesis", and "repair replication". In the following discussion the term repair replication will be used.

Repair replication was first studied in bacteria where it was shown by Pettijohn and Hanawalt (1) that the process probably involved a partial degradation of the damaged DNA followed by a resynthesis of the degraded portion. This interpretation was subsequently confirmed by other workers who also showed that, in the case of UV damage, the photoproducts formed in DNA were released from the molecule concomitant with repair replication (2,3). The biological significance of repair replication in bacteria is supported by studies of mutant strains which have been found to lack one or more of the enzymatic activities associated with the process. These strains are characterized by an extreme sensitivity to radiation (4).

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Biochemical evidence that a process similar to the bacterial repair replication was also present in mammalian cells was first reported by Rasmussen and Painter (5,6). Mammalian cells normally double their DNA content in preparation for division during a specific period in their growth cycle (S-phase). However, after exposure to UV light, x-rays, or certain chemicals, mammalian cells will incorporate exogenous DNA precursors into macromolecular DNA whether they are in the S-phase or not. This can be demonstrated by exposing a cell culture to the damaging agent and then labeling the cells with a radioactive DNA precursor such as ^3H -thymidine (^3H -dT) for a short period. Radioautographs of untreated cells thus labeled will show only a fraction of the cells with silver grains over the nuclei, representing incorporation of the label during normal DNA synthesis. Radioautographs of the treated cells will show incorporation of the label into all cell nuclei, including cells in mitosis. Experiments patterned on those performed with bacteria showed that this special DNA synthesis is different from the normal, semiconservative DNA synthesis which doubles the DNA content of the cell prior to division. As in the bacterial system, the evidence indicates that exogenous DNA precursors are incorporated into relatively short, single-stranded regions of the DNA double helix (6).

Since the first reports of repair replication in mammalian cells, the phenomenon has been studied in a number of laboratories. The results of these studies have indicated that mammalian cells possess a repair system that is capable of recognizing a number of different kinds of damage and, presumably, attempting repair. The hypothetical repair system consists of at least three parts: An endonuclease which recognizes a defect in DNA, breaks the DNA chain and excises the damaged region; A DNA polymerase which replaces the excised region with the proper sequence, presumably using the opposite strand as a template; A ligase or sealase which makes the final phosphodiester link between the newly-inserted nucleotides and the end of the previously existing DNA strand (6,7,8). The net result is a "cut-and-patch" repair. On the molecular level, we have shown that once the DNA has been "repaired" it can then replicate in a semi-conservative manner as required for normal DNA duplication (9).

Whether or not repair replication in mammalian cells is biologically significant has not been unequivocally proven. However, there is strong circumstantial evidence to support this contention. In humans, the lack of the complete repair replication system is associated with extreme sensitivity to UV light and a predisposition to skin cancer in those individuals exposed to sunlight. Persons with the hereditary disease xeroderma pigmentosum must be protected from sunlight or they develop skin lesions and in many cases skin cancer. In cultures of skin cells from these people, DNA repair replication is absent or defective (10). This finding has been confirmed *in vivo* (11).

Chemicals that Affect DNA Repair. In attempts to establish the nature of DNA repair replication, a number of inhibitors of DNA synthesis have been tested for their effect on DNA repair. The general findings have been that some compounds inhibit both normal and repair replication, while others inhibit only normal replication (12,13,14). There is no evidence known to this writer for the specific inhibition of DNA repair by any chemical agent. However, since only a relatively small number of compounds have been tested, it is premature to draw any general conclusions. It has been recently reported that several compounds inhibit DNA repair synthesis in unstimulated human lymphocytes (15,16). In this system, DNA repair synthesis is apparently possible, but normal DNA synthesis only occurs after the cells have been treated with a mitogen such as phytohemagglutinin. Hence the effect of the test materials on normal DNA synthesis was not measured. Nevertheless, it is of interest that the cocarcinogens phorbol ester and anthralin inhibited repair replication (16). Therefore, it is important that the effects of these compounds be studied in a cell system that is susceptible to the effects of chemical carcinogens.

Aryl Hydrocarbon Hydroxylases. The mixed-function oxidases or aryl hydrocarbon hydroxylases (AHH) are induced in mammals by a wide variety of chemicals including therapeutic drugs, steroid hormones, and carcinogens (17,18). They are especially important in regard to chemical carcinogenesis because it is generally believed that this group of enzymes is responsible for the activation or conversion of many carcinogens to the ultimate carcinogenic form that then reacts with cellular material to cause malignant transformation (19,20). In our laboratory we have studied the action of the AHH enzymes on the carcinogen benzo[a]pyrene (BP) in cell cultures, organ cultures, and in cell-free preparations from a variety of tissues (21,22,23). We have found that the specific metabolism of BP is characteristic of the species and tissue, and also may be modified by treatment of the animal or cell cultures with various compounds (23,24,25). Of particular interest for the proposed studies, we have found that at least two metabolites of BP, the 4,5-epoxide and another as yet unidentified form, can react *in vitro* with DNA to give rise to an apparent covalent linkage (22,25). Others have presented similar findings with a variety of different carcinogens that are activated by the AHH enzymes (26,27,28,29). Therefore, it is clear that the AHH enzymes are responsible for the binding of many carcinogens to DNA, and consequently may affect DNA repair. For this reason we feel that it is important to examine the effects of the compounds to be tested on the AHH enzymes, both in terms of induction of enzymatic activity and also the specificity of the enzymatic reactions.

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METHODS OF PROCEDURE

Compounds to be Tested for Their Effect on DNA Repair. Because of its potent action as a cocarcinogen and tumor promoter, the first compound to be tested will be phorbol ester. As noted above, it has been reported as an inhibitor of DNA repair synthesis in human lymphocytes (16). Additional test compounds will include representatives of the materials present in tobacco smoke or as environmental pollutants from other sources. Examples of toxic materials would be CN^- , CO, acetaldehyde, and acrolein. Carcinogenic and noncarcinogenic hydrocarbons are known to physically associate with DNA and thus deserve testing. Other radiation-sensitizing drugs (chloroquine, quinacrine) which have been reported to inhibit DNA repair in human lymphocytes (15) should also be tested in the proposed experiments. It is hoped that, within a few months, fractions of tobacco smoke condensates will become available for testing. However, until that time, there are many individual components that are available as off-the-shelf chemicals.

Cell Type to be Used. NIH-Swiss mouse embryo cells will be used in the first series of experiments. It is planned to obtain the cells as primary cultures from Microbiological Associates, and to use them in the experiments at the first or second passage. In this way we hope to minimize variability in the experimental cultures, and to avoid possible changes that may occur with repeated passage in culture. The cells will be grown in disposable plastic flasks and dishes using Eagle's minimum essential medium supplemented with fetal calf serum.

Depending upon the results with the mouse cells, the experiments may be extended to include Syrian hamster embryo cells, which are also susceptible to in vitro transformation by chemicals.

Cell Toxicity Studies. In order to carry out experiments on the effects of particular compounds on DNA repair it will be necessary to determine the highest concentration of test compounds that will still allow cell survival, so that there will be the greatest chance for detection of possible effects on DNA repair. Furthermore, in order to determine whether the effect of a particular compound is specific for DNA repair or also affects normal DNA synthesis, it will be necessary to know the effects of the test materials on normal DNA synthesis. Therefore, the toxicity studies will measure 1) the rate of cell proliferation as indicated by the cell doubling time and 2) the rate of DNA synthesis as measured by the incorporation of 3H -dT into macromolecular DNA when the cultures are exposed to the test compounds.

Cell proliferation studies will be done by seeding the cells in 60 mm plastic dishes at a concentration (approx. $2-4 \times 10^5$ cells per dish) that will allow for 3-4 cell doublings before the cell layer becomes confluent. The test compounds will be added to the cell growth medium at various concentrations, and, at intervals, the number of cells in sample dishes will be determined by hemacytometer count. One or two hours prior to harvesting the sample cultures, 3H -dT will be added to the culture dishes in order to label the DNA. Upon harvesting the cells, a portion of them will be processed to determine the extent of incorporation of 3H -dT into macromolecular DNA. This will be done in a manner similar to that described by Scott, et al. (30). Briefly, the method involves digestion of the cells with 1 N NaOH which hydrolyses the RNA, precipitation of the nucleoprotein by the addition of HCl, and treatment of the washed precipitate with hot perchloric acid which hydrolyses the DNA. Measurement of the absorbance of the perchloric acid extract at 265 nm, and determination of the radioactivity in a liquid scintillation spectrometer will indicate the specific radioactivity of the DNA. The latter will then be an index of the rate of DNA synthesis in the treated cells.

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Parallel cultures containing glass coverslips on which the cells will have grown will also be labeled with ^3H -dT. At the end of the labeling period, the coverslips will be processed for radioautography. This will allow measurements of the number of cells in S-phase, and, together with the specific radioactivity measurements, will enable the determination of the absolute rate of DNA synthesis, i. e., the rate per cell in S-phase. When compared with the appropriate controls, these experiments will show the effect of the test compounds on normal DNA synthesis. If the test compounds themselves induce DNA repair, this will be apparent in the radioautographs (see below).

DNA Repair Studies. In the proposed experiments the DNA repair process will be stimulated in the cell cultures by exposure to UV light at 245 nm. This choice was made because UV light at this wavelength produces a specific kind of damage to DNA (pyrimidine dimers), and the subsequent DNA repair can be readily quantified.

DNA repair replication can be assayed in two ways. Radioautographic techniques can be used to demonstrate repair replication in cell cultures after exposure to radiation or chemicals. In this method, the cell cultures are labeled with high specific activity ^3H -dT after treatment with the damaging agent. Radioautographs are prepared, and after exposure and development, repair replication can be quantified by counting the silver grains in the emulsion overlaying the cell nuclei (6). Cells participating in repair replication can be distinguished from those in normal DNA synthesis at the time of labeling because the rate of repair replication is of the order of 1% of the rate of normal DNA synthesis. Therefore, by proper choice of labeling time and exposure time, cell nuclei undergoing repair replication will appear lightly labeled with a countable number of silver grains while those in normal DNA synthesis will appear completely covered.

An alternate method of measuring DNA repair involves the use of CsCl gradient techniques and high specific activity ^3H -bromodeoxyuridine (^3H -BrdU), an analog of thymidine. During normal DNA synthesis, ^3H -BrdU is incorporated into new DNA strands in place of thymidine and, because of the presence of the bromine atom, the new DNA has a higher buoyant density than normal. (32). By centrifuging the isolated DNA in a concentrated solution of CsCl, new DNA can be separated from that which did not replicate during the period of labeling with ^3H -BrdU. During centrifugation, a concentration gradient of CsCl forms in the centrifuge tube, and the individual DNA molecules will come to equilibrium in this gradient at a position where their buoyant density equals that of the CsCl solution. Nearly all of the radioactivity is found associated with the newly made DNA of high buoyant density. As discussed above, repair replication appears to involve the degradation and resynthesis of relatively short, single-stranded regions of DNA. When ^3H -BrdU is used as a label following treatments which damage DNA in cell cultures, and the DNA is extracted and analysed as described, radioactivity is found associated with both the newly-made DNA and also with the unreplicated DNA (6,9,33). The conclusion is that ^3H -BrdU is incorporated into DNA during repair replication, but the amount incorporated is too small to significantly change the buoyant density of the isolated DNA molecules. Therefore, using this method, one can assay repair replication by measuring the specific radioactivity of unreplicated DNA that has undergone repair replication.

Both of the assay methods described would be used in the proposed studies.

AHH Studies. In the proposed experiments the AHH enzymes in the cell cultures would be assayed using the methods that we have developed in conjunction with other studies in this laboratory. These techniques were adapted from those described earlier by Nebert and Gelboin (34,35), and Grover and Sims (36). Briefly, the procedure involves exposure of the cell cultures to the test compound for several hours, followed by the preparation of microsomal fraction or homogenate of the cells. The cell-free preparation then serves as the source of AHH activity, which is assayed under standardized conditions. In the proposed studies, ^3H -benzo[a]pyrene would be used as

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the substrate for the AMH enzymes, and analysis of the products would be done using thin-layer chromatography. This would allow the quantification of metabolic products, and also the detection of possible changes in the spectrum of products as a result of the experimental treatment. The details of this procedure will be found in the accompanying reprints (Wang, et al., BBRC 49:1142, 1972., Borgen, et al., J. Med. Chem. 16: 502, 1973).

Estimated Experimental Schedule. The estimated time for completion of the studies for each of the compound to be tested is 6-8 weeks. However, since some of this time represents the exposure time for the radioautographs, it will be possible to work with several (i. e. 4-6) compounds simultaneously. Therefore, in the first year we can expect to have data on about 40 compounds.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Laboratory space of approximately 600 ft² is now assigned to the applicant for use in these and related studies. The laboratory is equipped with a fume hood for use with volatile or toxic materials.

Major Equipment:

Packard TriCarb liquid scintillation spectrometer
Beckman-Spinco Model L preparative ultracentrifuge
MAPCO CO₂ Incubator
Beckman Model DU Spectrophotometer

Other facilities available on a shared basis include a darkroom and walk-in cold room.

11. Additional facilities required:

None

12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

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14. First year budget:

A. Salaries (give names or state "to be recruited")
Professional (give % time of investigator(s)
even if no salary requested)

Rasmussen, Ronald E., Ph.D.

% time

Amount

50%

\$10505

Technical

Staff Research Associate I (to be recruited)

100%

\$10971

Sub-Total for A

\$21476

B. Consumable supplies (by major categories)

Radioactive Compounds

\$ 1600

Disposable plastic ware

1000

Glassware

300

Photographic emulsion for radioautography

300

Cell cultures and cell culture medium

2600

Chemicals, including test cmpds., CsCl, NADPH, etc.

1000

Sub-Total for B

\$ 6800

C. Other expenses (itemize)

Radioactive waste disposal

\$ 150

Equipment maintenance contracts

300

Estimated equipment repairs

200

Medical illustration, Publications

500

Travel for R. E. Rasmussen

500

Misc., office supplies, etc.

200

Sub-Total for C

\$ 1850

Running Total of A + B + C

\$30126

D. Permanent equipment (itemize)

None

Sub-Total for D

-0-

E

\$ 4519

Total request

\$34645

E. Indirect costs (15% of A+B+C)

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	\$23000	\$7000	\$2000	-0-	\$4800	\$36800
Year 3						

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16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Chemical carcinogenesis and mammalian DNA repli- cation.	U.S.P.H.S. CA-11939-03	\$36,768	6/1/73-5/31/74

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Metabolism of Carcinogens in animals and humans	U.S.P.H.S. CA-11939-04	\$56,068	6/1/74-5/31/75
Environmental pollution and carcinogen metabolism	U.S.P.H.S. ES-CA-01070	\$34,359	7/1/74-6/30/75

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Ronald E. Pasquissen, Ph.D.

Signature Ronald E. Pasquissen Date 1/28/74

Telephone 415 666-1041
Area Code Number Extension

Checks payable to

Reagents of the University of California

Responsible officer of institution

Mailing address for checks
Gifts & Endowments Office
University of California
San Francisco, California 94143

Type Sue Clark
Program Coordinator
Title Gifts and Endowments

Signature Sue Clark Date 1/31/74

Telephone 415 666-2047
Area Code Number Extension

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UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

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SANTA BARBARA • SANTA CRUZ

CHARLES J. HITCH
President of the University

FRANCIS A. SOOY, M.D.
Chancellor at San Francisco

University Relations
1487 Fourth Avenue
San Francisco, California 94143
January 31, 1974

The Council for Tobacco Research
110 East 59th Street
New York, New York 10022

Gentlemen:

We are presenting for your review, a request for support of the following project:

PROJECT TITLE Effect of Cocarcinogens and Tumor Promoters on DNA
Repair in Mammalian Cells Susceptible to Chemical
Transformation

PRINCIPAL INVESTIGATOR Ronald E. Rasmussen, Ph.D.

TYPE OF PROPOSAL New

AMOUNT REQUESTED

First 12 months	\$30,126.00	(indirect cost not included)
First 12 months	\$34,645.00	(including indirect cost)
Total period	\$71,445.00	(including indirect cost)

INDIRECT COST RATE USED 15% Total direct cost exclusive of per-
manent equipment

PROJECT PERIOD

First 12 months	4/1/74 - 3/31/75
Total period	4/1/74 - 3/31/76

Your favorable consideration will be appreciated. If the request meets with your approval, or if you have any questions, please communicate with this office.

Sincerely yours,

Sue Clark

Sue Clark
Program Coordinator
Gifts and Endowments

SC:ed

Enclosures

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#950 - ROSENKRANZ

1003545267

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

January 13, 1974

Grant application No. 950

CANCER

To: The committee comprising Drs. Andervont, Huebner and Meier

Subject: Herbert S. Rosenkranz, Ph.D., Columbia University, New York
New application No. 950

"Study of the Potential Mutagenicity of Cigarette Smoke
Condensates"

Basic method by Ames @ Berkeley

History

This proposal was Case No. 209, and formal application was encouraged.

Request

Application No. 950 requests \$14,134.00 plus one additional year.

Documents Received (Attached)

1-Application, undated, received by CTR December 29, 1973 (16 pages).

2-CV of Dr. Rosenkranz and list of 142 publications.

3-CV of Dr. Speck with list of his publications.

*Clinton to collect
Urine specimens
Ames at Berkeley*

Comment

We shall seek evaluation by an outside consultant.

FWN:gh

FWN
F.W.N.

Enclosures

1003545268

THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022

(212) 421-8885

Application for Research Grant

(Use extra pages as needed)

DEC 27 1973

Date:

1. Principal Investigator (give title and degrees): Herbert S. Rosenkranz, Ph.D.
Professor of Microbiology

2. Institution & address: College of Physicians and Surgeons
Columbia University
630 West 168 Street
New York, New York 10032

3. Department(s) where research will be done or collaboration provided:

Department of Microbiology

4. Short title of study:

STUDY OF THE POTENTIAL MUTAGENICITY OF CIGARETTE SMOKE
CONDENSATES

5. Proposed starting date: July 1, 1974

6. Estimated time to complete: 1 year

7. Brief description of specific research aims:

- A) The mutagenicity of cigarette smoke extracts and fractions derived therefrom will be investigated. (Preliminary results indicate the presence of mutagens in such extracts).
- B) Attempts will be made to identify the nature of the substance(s) giving a positive test.
- C) The presence of mutagens in the urines of smokers (and non-smoker controls) will be investigated.
- D) The ability of tissue extracts to enzymatically activate substances present in cigarette smoke extracts will be investigated.

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8. Brief statement of working hypothesis: It is well recognized that many known carcinogens are mutagens as well. Therefore screening for mutagens can be used to detect potential carcinogens.

The procedures for detecting mutagens are rapid, simple and relatively inexpensive. They are, therefore, well-suited for the project described herein.

9. Details of experimental design and procedures (append extra pages as necessary)

INTRODUCTION

The effects of environmental agents on human health is a subject of considerable concern at the present time (1, 2) especially as the use of chemical pollutants is on the increase (3-5). Of special concern to health scientists are the long-term effects of such environmental agents and more specifically their possible carcinogenicity, mutagenicity and teratogenicity. It has been estimated that at least 75% of all human cancers are due to environmental factors (6). Recently atmospheric extracts have been shown to be oncogenic in several systems (7-9, for additional references see ref. 7; for an analysis of the components of the atmosphere, see ref. 10). Ideally all environmental agents should be assayed in animals to determine their potential genetic and carcinogenic effects. Such an undertaking would however be time-consuming and very expensive and would possibly be beyond our national resources at the present time. Moreover to obtain reliable results that could be extrapolated to the human population, the number of animals required per group would have to be 30,000 rather than the 50 or 100 which are used currently (11). Furthermore, there is the added complicating factor of species specificity, agents affecting one species may have no effect on another. Thus 2-naphthylamine is a potent carcinogen

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9. (continued)

for man but it is without effect on the rodents commonly used in testing for carcinogenicity (12). Therefore, even if it were economically possible to test all environmental agents in animals, such a screening is not always foolproof. It has been suggested (1) that microbial systems capable of detecting mutagens and potential carcinogens be developed and used in screening. Indeed a national effort in this direction is currently being organized by the Division of Cancer Cause and Prevention of the National Cancer Institute and the applicant is a participant in that study. Substances that give positive tests in these microbial assays will then be tested in animals, while such tests are underway, the suspected agent could possibly be withdrawn from general use or its use could be regulated.

It is the applicant's experience that two microbial assays lend themselves to an investigation of the potential mutagenicity of substances present in tobacco smoke condensates. Indeed the applicant has obtained preliminary data suggesting the feasibility of such a study (see below). In view of the known relationship between mutagenicity and carcinogenicity (13-16), it is believed that results obtained by the procedures outlined will have a bearing on the health hazard associated with cigarette smoking.

EXPERIMENTAL PROCEDURES

Mutagenic Effects:

A powerful system for detecting mutagens and carcinogens was introduced recently by Ames (17,18). The procedure is quite simple and involves placing the mutagen at the center of a Petri plate containing minimal medium that is seeded with bacteria (*S. typhimurium*) unable to grow because of a deficiency in their histidine biosynthetic pathway. Revertants to histidine-independence are seen as colonies in a ring around the area on which the agent has been deposited. After testing a large number of mutants, Ames selected several strains with low spontaneous

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reversion rates and high sensitivity to various agents. Each of the strains represents a different class of mutants: a) strains which detect mutagens that cause base-substitutions, they revert either by direct mutation or by suppressor mutations and b) strains capable of detecting frameshift mutations.

This procedure appears to be capable of detecting all sorts of mutations and it may well be the most comprehensive of the procedures available to date. This method has been used not only by Ames but by others as well to detect and identify environment mutagens and carcinogens (see for example refs. 17-25). A modification of the procedure in which the test substance is incorporated into a soft agar overlay has also been developed (19). The results obtained by this procedure are more quantitative since they do not depend upon the rate of diffusion of the test substance and it is especially useful for detecting poorly diffusible substances (25).

Microorganisms Deficient in DNA Repair

"Normal" cells exposed to noxious agents which alter the cellular DNA will attempt to overcome this effect by excising portions of the modified DNA and resynthesizing the correct sequence. The enzyme DNA polymerase I has been implicated in this repair process (both in the repair replication step and in the excision step in excision repair) (26-32). It is to be expected, therefore, that cells lacking this repair enzyme will be more sensitive to the action of agents which react with cellular DNA. The recent availability (27) of *E. coli* mutants (pol A_1^-) lacking this enzyme, has permitted verification of the prediction. Using normal (pol A^+) and DNA polymerase-deficient (pol A_1^-) strains of *E. coli*, it was shown that pol A_1^- was much more sensitive than pol A^+ to a large number of agents known to alter cellular DNA, these included known mutagens and carcinogens (27, 30, 31, 33-40).

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Based upon these observations a simple assay procedure was devised in this laboratory (38). Bacteria (pol A^+ and pol A_1^-) are spread onto the surface of agar plates and discs containing the substance to be tested are placed on the plates. After incubation (7-16 hours) the diameter (or areas) of the zones of inhibition are measured. Agents known to alter cellular DNA were found (38) to produce larger zones of inhibition on the pol A_1^- plates than on the corresponding pol A^+ ones. Agents known not to alter the cellular DNA (e.g. cycloserine, chloramphenicol, methicillin, etc.) gave equal zones of inhibition on both sets of plates. No conclusions could be drawn for substances which inhibited neither strains as this could be due either to inertness of the substance, or inability to penetrate into the cell or finally it could reflect the fact that the test substance normally requires metabolic activation but that this is beyond the metabolic machinery of the bacterium (but see below).

Using this procedure, the applicant and his collaborators (41-48), as well as others (49) tested a number of environmental agents some of which gave positive results. Since it is probable that intercalating agents (frameshift mutagens) are an important class (19) of environmental agents (e.g. polycyclic hydrocarbons intercalate in DNA (50)) it may be of significance that nitrosofluorene and N-hydroxylaminofluorene gave positive results in this system (38).

Metabolic Activation of Mutagens and Carcinogens

In the bioassay system using E. coli pol A_1^- and Salmonella (described above) it was found that a number of known carcinogens (e.g. dimethylnitrosamine, diethylnitrosamine, 4-aminobiphenyl, etc. (25)) were completely inactive. Presumably this was a reflection of the fact that these substances are not the ultimate carcinogens and they require metabolic activation by mammalian enzymes (51-56). Such activation is beyond the metabolic capability of microorganisms (57).

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To overcome these problems extracts from rat liver with certain added co-factors were incorporated into the plate assays and this permitted the determination of the preferential inhibition of the pol A_1^- by dialkyl nitrosamine (38) as well as by a number of aromatic amines (25). More recently it was found that better results could be obtained if a mixed extract derived from rat and hamster livers was used (25). A similar modification (16) of the mutagenesis assay using Salmonella has permitted the demonstration of the mutagenicity of a number of carcinogens which are known to require metabolic activation (16, 25).

PRELIMINARY FINDINGS:

A series of cigarette smoke condensates as well as fractions derived therefrom were kindly provided by the Agricultural Research Service. Upon analysis in the DNA polymerase-deficient (pol A_1^-) E. coli system it was found that some of these substances preferentially inhibited the growth of the pol A_1^- strain (Table 1). This is a property they shared with the known mutagens and carcinogens propane sultone, methylnitrosanitroguanidine, β -propiolactone, 4-hydroxylaminoquinoline-N-Oxide (Table 1) and others. Agents known to react with cellular sites other than the DNA inhibited the parent (pol A^+) and pol A_1^- strains to the same extent; e.g. chloramphenicol, methicillin and streptomycin (Table 1).

The effect of the cigarette smoke condensates on the Salmonella mutagenesis system was also investigated. The procedure for detecting revertants to histidine-independence was discussed above. The Salmonella typhimurium strains used TA 1530, TA 1535 and TA 1538 were kindly provided by Dr. Bruce N. Ames. Strains TA 1530 and TA 1535 are essentially isogenic, they have a base-pair change in the histidine G gene and can be used to detect mutagens causing base-pair

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co-factors were incorporated into the assays and this permitted the

Determination of Effect of Cigarette Smoke Condensates on DNA Polymerase-Deficient Bacteria

(30) as well as a number of aromatic amines (25)

Substance	Amount (mg)	Diameters of Zones of Inhibition	
		Pol A ⁺	Pol A ₁ ⁻
Crude Tar	2.6	8.1	11.6
Benzene Extract	1.3	0(6.35)	8.0
Fraction No. 2	5.3	11.1	15.2
Fraction No. 9	1.5	7.8	11.1
Fraction No. 13	3.8	8.3	13.7

Propane sulfone	250 µg	11.9	18.9
N-Methyl-N-Nitroso-N'-Nitroguanidine	250 µg	25.0	33.8
β-Propiolactone	2 µl	12.3	20.2
4-Hydroxylaminoquinoline-N-Oxide	250 µg	7.7	11.1

Chloramphenicol	30 µg	28	28
Methicillin	30 µg	29	29
Streptomycin	10 µg	19	19

(Table 1)

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changes. However strain TA 1535 differs from TA 1530 in that it is a deep-rough strain deficient in cell envelope lipopolysaccharides which increases its permeability to large molecules. Strain TA 1538 has a mutation in the D gene of the histidine operon, it is also a deep-rough strain. It can be used to detect frameshift mutations. In the assay that was used known quantities of test substances were incorporated into the agar overlay together with the test bacteria.

Our preliminary results, which are the averages of duplicate determinations indicate that the cigarette smoke extracts specifically increased the rate of mutation of strain 1538 (Table 2). This is taken to mean that the cigarette smoke extracts contained mutagens which caused frameshift (but not base-substitution) mutations. Frameshift mutations are generally caused by planar molecules capable of intercalating between base-pairs of the DNA double-helix. This leads to the prediction that the substance(s) responsible for the positive test may be aromatic in character. (The reliability of the assay was tested by using methylmethanesulfonate (MMS) and 4-hydroxylaminoquinoline-N-Oxide (HAQNO) as controls (Table 2). The former causes only base-substitutions while the latter induces frameshift mutations. In fulfillment of this prediction it was found that MMS induced mutations only in strains TA 1530 and TA 1535 while HAQNO mutated strain TA 1538 only (Table 2)).

RESEARCH PROPOSAL

I. The results summarized in Tables 1 and 2 are very promising and they certainly warrant repetition. It is proposed, therefore, to repeat the assays using a new batch of cigarette smoke extracts and fractions derived therefrom.

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Changes, however small in 1535 differs from TA 1538 in that it is a

roughly equal in its behavior - hydrophobicity, etc.

its permeability to large molecules. Strain TA 1538 is more permeable to large

Table 2

INDICATION OF POSSIBLE MUTAGENICITY OF CIGARETTE SMOKE CONDENSATES

Fraction	μg	Revertants per Plate		
		TA 1530	TA 1535	TA 1538
Control (Acetone)	0	21	27	11
CRUDE TAR	26.4	22	20	15
Benzene Extract	13.0	24	27	26
Fraction No. 2	52.5	23	24	25
Fraction No. 9	15.3	22	24	23
Fraction No. 13	38.2	22	22	16
Fraction No. 14	0.6	22	22	16
Methylmethanesulfonate	10 μl	63	41	10
4-Hydroxylaminoquinoline-N-Oxide	1 μg	17	20	40
Water	10 μl	20	22	11

Cigarette products and the other test substances were incorporated into the soft agar layer and plates incubated in the dark for 48 hours.

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The data of Table 2 suggest that there is some specificity to the substance(s) responsible for the mutagenicity, i.e. only the strain indicative of frameshift mutations was affected. However due to a shortage of material, only low levels of cigarette smoke extracts could be tested and accordingly the number of mutants obtained was small. When more material becomes available the assay will be repeated. This should enable us to get more meaningful numbers and to ascertain whether other types of mutations (i.e. base-substitutions) can be detected when the amount of test material is increased.

II. Should, as expected, the above experiments be reproducible, then it will be possible to test cigarette smoke extracts derived from a variety of tobaccos and also from different parts of the tobacco plants.

III. As has been mentioned above, the bioassays used are simple and rapid.

It is feasible therefore to use them to monitor the isolation of the active principle. Accordingly, efforts will be made to purify the activity principle by solvent extraction and chromatographic procedures in order to establish the nature of the active principle.

IV. Many carcinogens require metabolic activation (see above) prior to exhibiting biologic activity in microbial systems. This ability to activate carcinogens is beyond the metabolic activity of bacteria. However by incorporating a mammalian cell-free extract into either the E. coli or the Salmonella system it is possible to activate these substances. Thus for example neither 4-aminobiphenyl nor 2-fluorenamine exhibit appreciable activity in the Salmonella system, yet when a rat liver extract is incorporated into the assay there is a tremendous increase in mutagenic activity (Table 3). It should be noted that the increase in mutagenic activity is specific for one strain only (Table 3).

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TABLE 3

responsible for the mutagenicity, i.e. only the strain indicator of frameshift

Salmonella Mutagenesis: EFFECT OF ACTIVATION *

Substance	Amount	Revertants per Plate					
		No Activation			With Activation		
		1530	1535	1538	1530	1535	1538
4-Aminobiphenyl	250 µg	22	34	13	27	33	140
2-Fluorenamine	250 µg	10	18	106	30	33	5655

*The test substance as well as the liver extract and required co-factors were incorporated into the soft agar overlay.

It is proposed, therefore, to determine whether the activity of cigarette smoke extracts in the E. coli and Salmonella system can be increased (or the specificity altered) by incubation in the presence of cell-free extracts derived from various tissues. Such a study should indicate which organ, if any, is involved in the metabolic conversion of tobacco derivatives.

V. Patients receiving cytoxan (cyclophosphamide) - therapy excrete a mutagen (an activated alkylating agent) in their urine. The activity of this substance can be demonstrated by the microbial assay procedures described above. It is proposed, therefore, to test the urines of heavy smokers (as well as of non-smoker controls) for the presence of a substance giving a positive test in the microbial bioassay systems. (Of course, care will be taken to select only individuals not receiving any medication and who are otherwise healthy). It may be necessary to concentrate the urines prior to testing. However, should positive urines be obtained, then the bioassay can be used to monitor the isolation of the active principle.

Significance of this Research:

The outline described above should help determine whether tobacco

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smoke condensates contain (a) substance(s) endowed with mutagenic (and therefore carcinogen) potential. If such (a) substance(s) exist(s) then the procedures described herein may help in identifying them and determining their chemical nature.

The proposed investigation may establish if the potential mutagenicity of cigarette smoke extracts is enhanced by cellular metabolic events and if so which organ is involved.

If the mutagenic agent is present in the urine of smokers, then it may be possible to correlate the amount of this material present to a) quantity of cigarettes consumed, b) the type of cigarette smoked, c) age, d) sex, e) state of health, medication taken, etc. The assay could also be used to determine for how long this substance is excreted after smoking is stopped. Obviously, many of these subsequent studies depend upon the earlier findings.

It is conceivable that these investigations could lead to the development of a "safer cigarette"; perhaps certain conditions will lead to a tobacco free of the mutagen. Moreover if the properties of the active principle are determined, it may be selectively extracted from the tobacco.

Experience of the Principal Investigator:

The applicant has first-hand knowledge of all the procedures described herein. The systems described are all in operation in his laboratory. A detailed curriculum vitae and a list of publications is appended hereunto.

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References

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

The laboratories in which these studies will be carried out are in the William Black Research Building, College of Physicians and Surgeons, Columbia University. These laboratories are equipped to carry out all of the procedures required for the completion of this research proposal. In addition, the Department of Microbiology, Columbia University, has good equipment for spectroscopic and radioisotopic determinations. The Department maintains an amino acid analyser, and an analytical ultracentrifuge equipped with Schlieren, ultraviolet and optical systems, microdensitometers, fluorescence microscopes, electron microscopes and accessory equipment, preparative centrifuges, constant temperature rooms and equipment for various chromatographic procedures. Facilities for tissue culture and bacteriological work are also available.

11. Additional facilities required:

NONE

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12. Biographical sketches of investigator(s) and other professional personnel (append):

Appended

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

14. First year budget:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s)
even if no salary requested)

Herbert S. Rosenkranz, Principal Investigator
William T. Speck, Co-Investigator

% time

Amount

20

20

—

—

Technical

1 Technician "to be recruited"
Fringe Benefits

100

8,500

1,190

Sub-Total for A

9,690

B. Consumable supplies (by major categories)

Glassware and media

1,600

Sub-Total for B

1,600

C. Other expenses (itemize)

Maintenance of Equipment
Cost of Publishing (Reprints, etc)
Office Supplies

400

400

200

Sub-Total for C

1,000

Running Total of A + B + C

12,290

D. Permanent equipment (itemize)

None

Sub-Total for D

1,843.50

E. Indirect costs (15% of A+B+C)

E

Total request

14,133.50

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	\$10,270	1,600	1,000	—	1,931	\$14,801
Year 3						

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16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE			
Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
<u>In vitro</u> and host-mediated Microbial Mutagenicity Assay Research Career Development Award	NCI NIH	\$65,135 Salary of	June 1973-June 1974 Principal Investigator 1965-1975
Gift	Marion Laboratories	\$27,000	per year
Gift	G.A. Carden Special Fund for Cancer Research	4,000	Unlimited

PENDING OR PLANNED			
Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Role of RNA in DNA Viruses	N.I.H.	\$20,000	?

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Herbert S. Rosenkranz

Signature Herbert S. Rosenkranz Date 11/21/73

Telephone 212 579-3658
Area Code Number Extension

Checks payable to

Trustees of
Columbia University

Responsible officer of institution

Typed Name Frederick B. Putney, Ph.D.

Title Assistant Vice President in Charge of
Medical Affairs

Signature Frederick B. Putney Date 11/21/73

Telephone 212 579-4148
Area Code Number Extension

Mailing address for checks

If awarded please mail check to:
Frederick B. Putney, Ph.D.
Assistant Vice President in
Charge of Medical Affairs

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#967 - ROSZMAN

1003545287

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

February 6, 1974

Grant application No. 967

CANCER

To: The committee comprising Drs. Gardner, Huebner and Meier

Subject: Thomas L. Roszman, Ph.D., University of Kentucky, Lexington
New application #967
"Effect of Substances Derived from Cigarette Smoke on the
Immune Response"

History

This proposal came to CTR as case #162 early in 1973. The Executive Committee decided to defer consideration for about one year.

Request

Application #967 requests \$29,824 plus two additional years.

Document Submitted

Attached is application dated 1/23/74 (14 pages).

Comment

The applicant states that this proposal is "completely different" from his research supported by the University of Kentucky Tobacco and Health Research Institute. He implies that senior staff of that Institute have encouraged this application, at least tacitly.

FWN:gh

F.W.N.

Encls.

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#967 a/c
1/31/74
JF

THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.

110 EAST 59TH STREET

NEW YORK, N. Y. 10022

(212) 421-8885

Application for Research Grant

(Use extra pages as needed)

Date: 1/23/74

JAN 31 1974

1. Principal Investigator (give title and degrees):

Thomas L. Roszman, Ph.D., Assistant Professor

2. Institution & address:

College of Medicine
University of Kentucky
Lexington, Kentucky 40506

3. Department(s) where research will be done or collaboration provided:

Department of Cell Biology

4. Short title of study:

Effect of Substances Derived from Cigarette Smoke on the Immune Response

5. Proposed starting date: July 1, 1974

6. Estimated time to complete: 3 years

7. Brief description of specific research aims:

The specific aims of this project are to investigate the effects of nicotine and the water soluble fraction from cigarette smoke on the immune response employing in vivo and in vitro models. More specifically, the proposed research is directed towards determining the effect of these substances on the: (a) in vitro and in vivo primary antibody response and (b) mitogen induced stimulation of murine lymphocytes.

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8. Brief statement of working hypothesis:

2.

Cigarette smoking may alter or suppress the immune response. If the immune response is suppressed, increased incidences of infection and cancer are noted. The experiments proposed in this study are directed toward determining whether or not water soluble substances derived from cigarette smoke are immunosuppressive. Both in vitro as well as in vivo systems will be employed to assess the immunosuppressive potential of these substances.

9. Details of experimental design and procedures (append extra pages as necessary)

As appended.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

The Department of Cell Biology is well equipped for the research to be undertaken in this proposal. Ample laboratory and office space is available. Available to the principal investigator are Zeiss spectrophotometers, refrigerated centrifuges, Spinco model L ultracentrifuges, liquid scintillation counters, fraction collectors, phase contrast microscopes, CO₂ incubators, cold rooms and electrophoresis equipment. Water baths, clinical centrifuges and a moderate supply of glassware are also available. The department has a central facility for washing glassware and media preparation. The Medical Center has a large central animal facility which is available.

11. Additional facilities required:

None.

12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

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14. First year budget:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s)
even if no salary requested)

Thomas L. Roszman
Fringe Benefits

% time

Amount

15%

2,730
382

Technical

Research Analyst (to be recruited)
Laboratory Aide (to be recruited)
Secretary (to be recruited)
Fringe Benefits

100%

35%

50%

9,300
1,462
2,500
1,060

Sub-Total for A

17,434

B. Consumable supplies (by major categories)

Glassware
Chemicals
Isotopes
Animals (1000 mice at \$1.40 each)
Tissue culture supplies (this includes CO₂
tanks, serum, plasticware, antigen, mitogens,
media and biochemicals)

800
600
500
1,400

2,000

Sub-Total for B

5,300

C. Other expenses (itemize)

Media room services
Travel (to attend FASEB meeting)
Publication costs (reprints and page costs)
Service contracts and maintenance
Office supplies (duplicating and photography)
Animal care (board costs of \$0.022/day/mouse)

400
300
200
500
200
1,600

Sub-Total for C

3,200

Running Total of A + B + C

25,934

D. Permanent equipment (itemize)

Sub-Total for D

--

E

3,890

Total request

29,824

E. Indirect costs (15% of A+B+C)

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	18,480	5,300	3,200	--	4,047	31,027
Year 3	19,600	5,300	3,200	--	4,215	32,315

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16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Analysis of IgM Memory	Research Corporation 203-65-7H400-T3916	3,980	4/1/72 - open
The Effect of Cigarette Smoke on the <u>In Vitro</u> Secondary Antibody Response	University of Kentucky Tobacco and Health Research Institute 124-05-7H400-24036	25,154	7/1/73 - 6/30/74

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
The Effect of Cigarette Smoke on the Immune Response	University of Kentucky Tobacco and Health Research Institute	34,800	7/1/74 - 6/30/75
Effect of Cigarette Smoke on the Humoral Immune Response	Lung Association	13,938	7/1/74 - 6/30/75

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Thomas L. Roszman

Signature Thomas L. Roszman Date 1/24/74

Telephone 606-233-5913

Area Code Number Extension

Checks payable to

University of Kentucky Research Foundation

Mailing address for checks

MN 145, A. B. Chandler Medical Center

Univ. of Kentucky, Lexington, Ky. 40506

Responsible officer of institution

Typed Name Carl B. Delabar

Title Associate Director, U.K.R.F.

Signature Carl B. Delabar Date 1-25-74

Telephone 606 233-5577

Area Code Number Extension

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9. Experimental design and procedures

A. Background and Rationale:

It is widely accepted that cigarette smoking is injurious to health. In the Surgeon General's report (1) on health and cigarette smoking, a convincing volume of statistical data has been compiled indicating that a relationship between smoking and certain diseases does exist. However, there is as yet little experimental data which document and substantiate the statistical data. In particular, there are few reports concerned with the effect of cigarette smoking on the immune response. Recent advances in immunologic knowledge and technology now make it possible to employ reliable in vitro as well as in vivo models for studying the effect of exogenous factors on the immune response. Thus, the purpose of this proposal is to examine the effects of products derived from cigarette smoke on the immune response.

The broncopulmonary system is an important portal of entry for antigen into the body and has the capacity to mount a local immune response (2). Environmental factors such as air pollution and cigarette smoking may not only damage the function of this system but the local immune response as well. An increased susceptibility to respiratory infection has been linked to cigarette smoking and air pollution (3, 4). Inhalation of cigarette smoke, furthermore, can result in the absorption into the circulation of soluble substances derived from smoke. A case in point is nicotine which reaches a blood level of about 50 ng/ml of plasma after the smoking of one cigarette (5). If derivatives of cigarette smoke can be disseminated throughout the body and attain sufficient levels in lymphoid tissues suppression may result. Recent reports indicate that cigarette smoke in fact has an adverse affect on the immune response. For example, young adult cigarette smokers had a significantly lower level of antibody to influenza virus A₂ after either natural infection or vaccination as compared to a similar group of nonsmokers (6). Thomas, et al. (7) have studied the effect of cigarette smoke on the primary and secondary antibody response of mice. Mice were exposed for 26 weeks to the smoke of 30 cigarettes daily and then inoculated intratracheally with sheep red blood cells. The number of direct and indirect antibody-forming cells found in the lungs, lymph nodes and spleens of smoked and unsmoked animals was determined on various days after primary and secondary immunization. The results demonstrated that the immune response of all organs obtained from smoked mice was substantially lower than those obtained from unsmoked mice. In particular, the primary immune response of the lungs was completely abrogated. The results also indicated that soluble substances derived from cigarette smoke were entering the circulation and attaining sufficient levels in the spleens and lymph nodes to cause immunosuppression in these lymphoid tissues. These same workers have also noted that the responsiveness of lymphoid cells from smoked mice to phytohemagglutinin is markedly depressed after 35 weeks of exposure to cigarette smoke (8). The in vitro functional activity of lymphocytes and macrophages is also decreased by cigarette smoke and extracts from smoke. Izard and co-workers (9) have found that human peripheral blood lymphocytes exposed to the gas phase of cigarette smoke have a reduced capacity to undergo stimulation with phytohemagglutinin. Similar observations have been reported by Savel (10). Macrophage function is also impaired after in vitro exposure to cigarette smoke. Thus, Green and Carolin (11) have demonstrated that cigarette smoke depresses the in vitro bacteriocidal activity of rabbit alveolar macrophages. These investigators have further demonstrated that water

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soluble extracts from the gas phase of cigarette smoke can inhibit the glyceraldehyde phosphate dehydrogenase activity of these macrophages establishing a relationship between the loss of a vital enzyme activity and the depressed bacteriocidal capacity of these cells (12). Similar studies suggest that nicotine also interferes with the function of certain enzymes particularly dehydrogenases (13). Nicotine, furthermore, can depress the respiration rate as well as the adenosine triphosphatase activity of alveolar macrophages (14).

Work performed in this laboratory has been concerned with establishing the effect of nicotine and the water soluble fraction (WSF) from the whole smoke of the University of Kentucky reference cigarette 1R1 on the in vitro secondary antibody response to sheep red blood cells (15). Cell suspensions prepared from the spleens of rabbits previously immunized with sheep red blood cells were treated with various concentrations of either nicotine or WSF before or after antigenic stimulation. The resulting IgM and IgG response was dependent on the concentration of either nicotine or WSF which was added to the lymphoid cell cultures. A progressive suppression of the IgM and IgG antibody responses was observed after treatment of these cell cultures with concentrations of nicotine and WSF ranging from 1 to 100 ug/ml. These concentrations of nicotine and WSF are not cytotoxic to the spleen cells. In all cases the addition to the cultures of either nicotine or WSF ranging in concentration from 200 to 1000 ug/ml induced complete suppression of both the IgM and IgG responses. Suppression of the IgM and IgG responses, furthermore, was observed after exposing the cultures to either nicotine or WSF for a period of 2 hr before antigenic challenge indicating that these substances have a rapid and irreversible effect on immunocompetent cells possibly as a result of interfering with the early events involved in the induction of the antibody response and not with the actual synthesis of antibody. The results of these studies as well as those of others previously described suggest that cigarette smoke and products derived from cigarette smoke have immunosuppressive potential and that cigarette smoking may in fact cause suppression of the immune response with resulting increases in the incidence of microbial infections and neoplasias.

From the foregoing discussion, it is apparent that further studies are needed to more clearly define the effects of cigarette smoke on all aspects of the immune response. Experiments are required to determine the mechanism(s) of action of cigarette smoke induced immunosuppression. Attention also needs to be focused on the effect of cigarette smoke on the different types of cells involved in the immune response since it is now well-established that thymus dependent lymphocytes (T-cells), thymus independent lymphocytes (B-cells) and in certain cases macrophages collaborate with one another to initiate the humoral immune responses (16, 17, 18). There is preliminary evidence which indicates that certain T-cell and B-cell functions are sensitive to cigarette smoke or substances derived from cigarette smoke (9, 10, 19).

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B. Method of procedure:

The effect of nicotine and WSF on the in vitro primary antibody response. The first series of experiments will be concerned with determining the effect of nicotine and the water soluble fraction (WSF) from the whole smoke of the University of Kentucky reference cigarette 1R1 on the in vitro primary antibody response of spleen cells obtained from adult B C3F₁ (C57BL X C3A/ANF) mice to sheep red blood cells (SRBC). For each experiment the mice will be matched to age and sex.

Freshly prepared samples of nicotine and WSF will be obtained from the University of Kentucky Tobacco and Health Research Institute Core Service Smoke Chemistry Laboratory. Mouse spleen cell suspensions will be prepared and the primary in vitro antibody response to SRBC will be performed according to the method of Mishell and Dutton (20). In brief, the procedure for carrying out the in vitro response will be as follows. Spleen cell suspensions will be adjusted to 1×10^7 nucleated cells/ml in Eagle's medium containing vitamins, glutamine, nonessential amino acids, antibiotics and 10% heat inactivated fetal calf serum (FCS). One ml volumes of the cell suspension will be placed in 35mm Petri plates and stimulated with 5×10^6 SRBC. The cultures will be incubated at 37C on a rocker platform oscillating at 10 cycles/min. Nutrient cocktail and FCS will be added to the cultures daily (20). At the time of addition of the SRBC and at 24 hr intervals thereafter, aliquots of the spleen cells will be removed to determine the number of direct (IgM) plaque-forming cells (PFC) employing a modification of the Cunningham assay procedure (21). Cell viability will be determined initially and at 24 hr intervals employing the trypan blue dye exclusion procedure.

Initial experiments will be involved with determining the kinetics of the in vitro primary antibody response to SRBC. After completion of these experiments, the effect of various concentrations of nicotine and WSF on the in vitro response will be assessed as follows. To duplicate antigen stimulated spleen cell cultures will be added from between 0.1 ug to 1000 ug/ml of either sterile nicotine or WSF. The spleen cell cultures will be exposed to these substances for the duration of the culture period. The number of PFC, percentage of viable cells and the number of nucleated cells recovered will be determined from day 0 through day 6. The data from these experiments will provide information with regards to the effect of nicotine and WSF on the time course and magnitude of the antibody response as well as the effect on cell survival.

Since nicotine and perhaps WSF are rapidly metabolized and eliminated under in vivo conditions, experiments will be designed to determine what effect short-term exposure to these substances will have on the in vitro primary antibody response of spleen cell cultures. Moreover, these experiments will provide information with regards to the effect of these substances on the inductive and proliferative phases of the response. The experiments will be performed and data collected as previously described except that the spleen cell cultures will be exposed to the various concentrations of either nicotine or WSF for a period of 2 hr prior to antigenic stimulation or at the time of antigenic stimulation and removed 24 hr later. The nicotine and WSF will be removed by washing the spleen cells three times with Eagle's medium containing 1% FCS and the cells finally resuspended to their original volume in complete medium containing 10% FCS and the other additives. Experiments will also be carried out to determine

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the effect of nicotine and WSF on the proliferative phase of the in vitro primary response. Either nicotine or WSF will be added to the cultures at either 24 hr or 48 hr after antigenic stimulation. Twenty-four hr later the cultures will be washed to remove these substances and the cells resuspended in medium and assayed for PFC as previously described.

Assuming that nicotine as well as WSF do have an effect on the in vitro primary antibody response other than simply a cytotoxic effect further experiments are proposed to determine the effect of these substances on the splenic macrophages and lymphocytes. It is now clearly established that the induction of the in vitro primary antibody response of mouse spleen cells to SRBC is dependent on the presence of macrophages (22, 23). Therefore, the mouse spleen cell suspensions will be separated into macrophage-rich and lymphocyte-rich populations according to the method of Mosier (22). Neither the macrophage-rich or lymphocyte-rich population alone in the presence of antigen will give rise to an in vitro primary response. However, recombination of these populations in the presence of antigen will yield a response. The basic premise of these experiments is to determine what effect nicotine and WSF have on these two separate and distinct cell populations. To perform these experiments either the macrophage-rich or lymphocyte-rich population will be exposed to either nicotine or WSF as previously described. Recombination experiments will be performed where, for example, treated macrophages will be added in sufficient numbers to a fixed number of untreated lymphoid cells in the presence of SRBC and the magnitude of the ensuing primary antibody response measured as previously described. Similar experiments will be performed employing treated lymphocytes which will be recombined with untreated macrophages. Suitable controls will be carried out to ensure that neither the macrophage-rich or lymphocyte-rich population alone will support a primary response but when combined will yield a response.

The effect of nicotine and WSF on the stimulation of murine lymphocytes by concanavalin A and lipopolysaccharide. If nicotine and WSF have an adverse affect on the lymphocyte-rich population as assessed from the results obtained with the in vitro primary response system, further experiments are proposed to determine whether or not these substances have a differential effect on T- and B-cells. It is well-known that lymphocytes can be stimulated with certain mitogens such as concanavalin A (con A) and bacterial lipopolysaccharides (LPS) to undergo DNA synthesis, blastogenesis and cell division. Moreover, LPS is specific for B-cells (24) where as con A has specificity for only T-cells (25). Cell suspensions will be prepared from the spleens of adult BC3F₁ mice. The cell concentration will be adjusted to 2×10^6 nucleated cells/ml in RPMI-1640 medium supplemented with 5% FCS, glutamine and antibiotics. One ml aliquots of the cell suspension will be pipetted into 12 x 75 mm plastic tubes and various concentrations of either nicotine or WSF ranging from 0.01 ug to 1000 ug added for various lengths of time as previously described. The cell suspensions will be stimulated with optimal concentrations of either con A or LPS. At 48 hr after the addition of the mitogens, one uc of H³-thymidine will be added to each culture and 18 hr later the radioactivity incorporated into DNA will be measured (26). The percentage of viable cells will also be determined by the trypan blue method.

The effect of nicotine and WSF on the in vivo primary antibody response. It is recognized that the in vitro effects of products derived from cigarette smoke on the primary antibody response do not necessarily indicate

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that similar effects will invariably occur in vivo. There are, for example, in vivo detoxifying mechanisms which are probably not operative in the in vitro system. A case in point is the very short in vivo half-life of plasma nicotine after the smoking of one cigarette (5) and it can be assumed that other substances derived from cigarette smoke may also be rapidly eliminated. Thus, it is important to determine the effect of nicotine and WSF on the in vivo primary antibody response to SRBC. Adult BC3F₁ mice matched to age and sex will be employed in these experiments. The acute toxicity of nicotine and WSF in mice will be determined initially to establish a range of suitable concentrations for use in the in vivo experiments. The LD50 will be determined by injecting from between 0.1 mg to 20mg/kg of either sterile nicotine or WSF in a constant volume into the lateral tail vein. Twenty mice will be used for each concentration of either nicotine or WSF. The concentration range to be employed for nicotine is predicated on the results of Stakandske (27) for NMRI strain white mice. No information is available on the WSF from the University of Kentucky reference cigarette 1R1 thus the dose range is empirical. Having established sublethal dose ranges of nicotine and WSF for BC3F₁ mice two basic types of experiments are planned. Groups of mice will be injected intravenously with a constant volume of various concentrations of nicotine and WSF either before or after immunization with SRBC as follows. Beginning 3 days before immunization and at 12 hr intervals groups of mice will be injected with various concentrations of either nicotine or WSF. Twelve hr after the final injection of these substances the mice will be immunized intravenously with 5×10^8 SRBC. In the case of those mice receiving either nicotine or WSF after immunization with 5×10^8 SRBC the following protocol will be used. Mice will be injected intravenously with various concentrations of either nicotine or WSF at either 24 hr or 48 hr after immunization. Control groups of mice will be similarly injected with diluent and SRBC. Five mice from each group will be sacrificed on days 4, 5, 6, 8, & 10 and the number of direct and indirect PFC found in the spleens determined (21). The data will be statistically analyzed to determine whether or not there are significant differences between the primary immune response of mice treated with nicotine and WSF and the response of untreated controls. These experiments will provide information on the question of whether or not nicotine and WSF interfere with inductive and proliferative phases of the in vivo primary antibody response.

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#966 - SLOANE

1003545299

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

February 6, 1974

Grant application No. 966

CANCER

To: The committee comprising Gardner, Huebner, Jacobson
Subject: Nathan H. Sloane, Ph.D., University of Tennessee
Medical Units, Memphis
New application No. 966
"Effect of Benzo(a)pyrene and Derivatives on Mammalian
Lung Cells"

History

A preliminary inquiry concerning this proposal was
handled as Case No. 254.

Request

Application No. 966 requests \$35,090 plus two additional
years.

Documents Submitted

Attached is application dated 1-24-74.

Copies of the papers checked on page 3c and 3e of the
application have been provided; they will be sent to you promptly
on request.

Also enclosed is a letter from Dr. Roy C. Page, dated
January 30, 1974, stating his readiness to provide human tissues
for the proposed study.

FWN:wg
Encls.

FWN
F.W.N.

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966

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8985

Application for Research Grant
(Use extra pages as needed)

JAN 31 1974

Date: 1-24-74

1. Principal Investigator (give title and degrees):

Nathan H. Sloane, Professor of Biochemistry, B.S., M.P.H., Ph.D.

Audrey N. Roberts, Professor of Microbiology, B.S., M.A., Ph.D.

2. Institution & address:

The University of Tennessee Medical Units
62 Dunlap Street
Memphis, Tennessee 38163

3. Department(s) where research will be done or collaboration provided:

Department of Biochemistry and Department of Microbiology

4. Short title of study:

Effect of Benzo(α)pyrene and Derivatives on Mammalian Lung Cells

5. Proposed starting date: July 1, 1974

6. Estimated time to complete: July 1, 1977

7. Brief description of specific research aims:

1. Determine whether metabolic hydroxymethylation of benzo(α)pyrene to the 6-hydroxymethyl derivative represents a pathway to form a more proximate carcinogen from the polycyclic hydrocarbon.
2. Determine the biological properties of benzo(α)pyrene carcinogenic polycyclic hydrocarbon derivatives in vitro using primary cultures prepared from human lung tissue, embryonic human lung tissue, rat lung tissues, and mouse lung tissues. These latter studies will utilize dwarf mutation mice (dw) and their normal sized litter mates.
3. Determine the effects of benzo(α)pyrene on primary and continuous cell lines in vitro in the presence and absence of cytochrome P-450 inhibitors using cytotoxicity and cell transformation as quantitative biological parameters.
4. Determine levels of the benzo(α)pyrene metabolizing enzymes in the lungs and the livers of the dwarf mice (which are tumor resistant) to correlate these enzymatic activities with tumor susceptibility.
5. Determine the binding of tritium labeled benzo(α)pyrene, 6-hydroxymethyl-benzo(α)pyrene, 6-methylbenzo(α)pyrene and 3-hydroxybenzo(α)pyrene to DNA, RNA and protein fractions of lung cells grown in culture in the presence and absence of cytochrome P-450 inhibitors.

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8. Brief statement of working hypothesis:

2.

Previous studies (NHS) have shown that the mammal possesses 2 metabolic pathways for the formation of aryl hydroxymethyl compounds: 1) oxidation of the aryl side chain methyl group to the hydroxymethyl group; 2) direct hydroxymethylation of the benzene ring - an aryl hydroxymethyl synthetase reaction. The synthetase reaction is independent of cytochrome P-450. However, the oxidation of the aryl side chain methyl group is cytochrome P-450 mediated. Furthermore, the hydroxylation of the benzene ring of polycyclic hydrocarbons also requires the participation of cytochrome P-450. Therefore, it is now possible to study the direct biological effect of the hydroxymethyl synthetase reaction of benzo(α)pyrene in the absence of the hydroxylation reaction. Theoretically the aryl hydroxymethyl compound should be a more proximate carcinogen as postulated by Dipple, Lawley and Brookes (Europ. J. Cancer 4: 493, 1968). Flesher and Sydnor showed that rat liver homogenates hydroxymethylate benzo(α)pyrene to the 6-hydroxymethyl derivative and these investigators also demonstrated that this derivative is carcinogenic. Sloane and Davis (manuscript submitted for publication) showed that the carcinogenic hydrocarbon is indeed hydroxymethylated by the soluble enzyme system obtained from liver microsomes.

9. Details of experimental design and procedures (append extra pages as necessary)

1. Study of the Enzymatic Hydroxymethylation of Benzo(α)pyrene

- a. We propose to prepare lung and liver homogenates in order to determine the concentration of the 6-hydroxymethyl synthetase. We shall utilize human tissues (lung and liver) as well as liver and lung of both the normal mouse and the dw litter mate that has been shown to be resistant to chemical carcinogenesis.

Bielschowsky and Bielschowsky (Brit. J. Cancer 13: 302, 1959; Brit. J. Cancer 14: 195, 1960; Brit. J. Cancer 15: 257, 1961) studied the effect of chemical carcinogens in the pituitary dwarf mouse. These investigators demonstrated that these mice were resistant to some carcinogens, namely 2-aminofluorene and dimethylbenzanthracene, whereas no resistance was shown by these mice to the carcinogen, methylcholanthrene. The hypophysectomized rat did show formation of sarcomas induced by benzo(α)pyrene (Zamurovitch, Onocolgia 6: 190, 1953).

- b. The microsomal fraction of the homogenate will be prepared and fortified with the above cofactors, boiled liver juice, and/or folate derivatives as described below.
- c. The soluble microsomal enzyme fraction of these microsomes will be prepared by the method of Sloane and Heinemann (Biochim. Biophys. Acta 201: 384, 1970). We have previously shown that hydroxymethylation of the benzene ring is accomplished by an enzyme system that is present in this soluble microsomal fraction; the aryl hydroxymethyl synthetase requires a reduced pyridine nucleotide and a macromolecule that donates the C-1 fragment.
- d. We shall study the possible role of folate derivatives on the hydroxymethylation of benzo(α)pyrene by the enzyme system. We shall investigate the effect of the addition of N¹⁰ formyl tetrahydrofolate (Robinowitz and Prieger, J. Biol. Chem. 237: 2898, 1962), N⁵ formyl tetrahydrofolate, N⁵ - N¹⁰ methylene tetrahydrofolate (the coenzyme of serine hydroxymethylase, thymidylate synthetase, and deoxycytidylate hydroxymethylase) to the enzyme system. The coenzyme, N⁵ - N¹⁰ methylene tetrahydrofolate will be prepared by the method described by Hunnekens,

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Ho, and Scrimgeour in *Methods in Enzymology*, Vol. VI, pp. 807-812, Academic Press, 1963).

- e. The enzyme will then be purified utilizing the conventional techniques of enzyme purification. These include separation of nucleic acids, ammonium sulfate fractionation, acetone and/or alcohol fractionation, ion exchange cellulose chromatography, Sephadex chromatography (G-25 to G-200) and/or Bio Gels to fractionate proteins according to molecular weights and DEAE-Sephadex anion exchange chromatography, and preparative gel electrophoresis. Evidence for the successful use of these conventional techniques of enzyme purification is provided in Publications of N. H. Sloane; 8,9,12,13,19,20,21,27 and 28.
- f. The hydroxymethylation of benzo(α)pyrene by the enzyme system will be determined by the isolation of the labeled product 6-hydroxymethylbenzo(α)pyrene by the use of thin layer chromatography as described by Flesher and Sydnor (63rd Annual Meeting of the Am. Assoc. for Cancer Research) utilizing the precursor compound that had been tritium labeled by catalytic exchange. The isolated labeled 6-hydroxymethylbenzo(α)pyrene will be counted so that we shall have a quantitative assay for this hydroxymethylation. This type of assay was successfully utilized in our previous studies on the hydroxymethylation of benzene.

Benzo(α)pyrene is commercially available, whereas the 6-hydroxymethylbenzo(α)pyrene has been prepared by the procedures of Flesher and Sydnor. It is not probable that methylation of benzo(α)pyrene precedes the formation of the hydroxymethyl derivative, because Sloane and Heinemann showed that S-adenosyl-L-methionine was not involved in the hydroxymethylation of benzene to benzyl alcohol.

We shall attempt to correlate the concentration of benzo(α)pyrene hydroxymethyl synthetase in the lung and liver of carcinogen susceptible and resistant dw mice as well as determine the concentration of the enzyme in normal human lung and neoplastic lung tissues. Dr. Roy Page of the Page Clinic (Memphis, Tennessee) will provide human tissues for these studies.

2. Study of the Enzymatic Hydroxylation of Benzo(α)pyrene

- a. We propose to determine the concentration of the benzo(α)pyrene hydroxylases (specifically 3-hydroxybenzo(α)pyrene in the tissue systems discussed above under the Section A. 1.a. These studies will be performed to correlate the concentration of the hydroxylase in the lung and liver of carcinogenic susceptible and resistant dw mice.
- b. The concentration of the hydroxylase will be determined by the method of Conney *et al.* (A. H. Conney, E. C. Miller and J. A. Miller, *J. Biol. Chem.* 228: 753, 1957).
- c. Since the hydroxylation of benzo(α)pyrene requires cytochrome P-450, we shall study the role of both mitochondrial and microsomal cytochrome P-450 on the oxidation reaction. During the summer months of 1969, 1970 and 1973, N. H. Sloane worked in the Laboratory of Population Sciences, Harvard University School of Public Health with Dr. H. A. Salhanick, on the isolation of cytochrome P-450 from Pseudomonas

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putida. We showed that P-450 CAM will not act as the steroid hydroxylating enzyme in beef adrenal mitochondria, in which the adrenal P-450 was destroyed by sonication. Furthermore, we showed that inhibitors of Ps. P-450 CAM (as evidenced by spectral changes) also inhibited the P-450 of bovine corpus luteum, thus indicating that specificity does not exist in the inhibitor binding sites.

- d. We have recently obtained evidence to indicate that the enzymatic activation of oxygen for the side chain hydroxylation of the aryl methyl group to the aryl hydroxymethyl group is mediated by a mixed function oxidase; the reaction studied was the conversion of toluene to benzyl alcohol. The hydroxylation reaction requires molecular oxygen, NADPH and cytochrome P-450. Evidence for the role of cytochrome P-450 for this oxidation was obtained using diverse cytochrome P-450 inhibitors. The compounds studied were carbon monoxide, metyrapone, 1-benzylimidazole and aminogluthethimide. These experiments employed the sonicate of rat liver microsomes prepared from 50 to 60 day old female Holtzman rats.
- e. The cell cultures to be studied will allow comparisons of carcinogen effects on normal and malignant human lung cells as well as on lung cells derived from normal and dwarf mice.

B. In Vitro Cell Culture Studies

1. Carcinogen Effects on Lung Cell Cultures

- a. The effects of benzo(α)pyrene, 3-hydroxybenzo(α)pyrene, k region epoxide of benzo(α)pyrene, 6-hydroxymethylbenzo(α)pyrene and 6-methylbenzo(α)pyrene will be determined in cell cultures initiated from normal and malignant human lung tissues, human embryonic lung and lung tissue from normal and dwarf mice. The cell culture techniques are described below. The effects of these compounds will be determined in the presence and absence of cytochrome P-450 inhibitors in order to delineate the role of the aryl hydroxymethyl synthetase reaction on the chemical carcinogenesis. The effects of the cytochrome P-450 inhibitors on the hydroxylation of benzo(α)pyrene will be determined by the method of Conney et al. (J. Biol. Chem. 228: 753, 1957).
- b. The effects of these inhibitors on the aryl side chain hydroxylation of 6-methylbenzo(α)pyrene to the 6-hydroxymethyl derivative will be determined by the method of Sloane and Davis (manuscript submitted for publication).
- c. Collection of normal and malignant human lung and human embryonic tissues. All human tissues will be provided through the courtesy of Roy C. Page, M.D. of the Page Clinic, Memphis, Tennessee. All normal and malignant adult tissue will be histologically examined by Surgical Pathologists as a part of routine surgical procedure to confirm normalcy and malignancy, respectively. Embryonic tissues will be obtained from first and second trimester human fetuses.

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- d. Cell culture techniques. --The human tissue specimens will be collected in Hanks' balanced salt solution (BSS) containing penicillin (50 units per ml), streptomycin (50 µg per ml) and mycostatin (40 units per ml) and transported to the laboratory on ice. All human and mouse tissues will be minced and washed with Hanks' BSS. Primary cultures will be initiated from tissue minces in 250 ml milk dilution bottles, planting 25 to 30 tissue fragments approximately two mm² per bottle. All cells will be cultured in Eagle's MEM containing 10 per cent fetal calf serum and antibiotics at 37 C under 5 per cent CO₂. When complete monolayers are obtained, the cells will be subcultured by treatment with EDTA alone or in combination with 0.025 per cent trypsin.

Carcinogen studies will be performed in 60 x 15 mm Falcon plastic petri plates seeded with approximately 1×10^6 cells per plate. Cover glass cultures to be used for autoradiography or routine morphological studies also will be grown as monolayers in petri plates.

- e. Cytochrome P-450 Inhibitors. Preliminary studies will determine the effects of cytochrome P-450 inhibitors on the plating efficiency and growth of each cell line. The inhibitors, aminogluthethimide, metyrapone, 4-phenylimidazole, 1-benzylimidazole and SKF 525A, will be tested at final concentrations of 1 to 2×10^{-4} M. For tests on plating efficiency, cells will be seeded in 5.0 ml of medium containing inhibitor and incubated for 24 hours at 37C under 5 per cent CO₂. The medium then will be withdrawn and the monolayers washed 2X with 5.0 ml of Hanks' BSS to remove unbound cells. The cells will be detached from the plastic surface with trypsin and counted in a hemacytometer. The number of cells present will be compared with the number from untreated cultures, both conducted in triplicate.

To study the influence of inhibitors on cell growth, the cells will be seeded in culture plates both in the presence and absence of inhibitor. Cultures seeded in the absence of inhibitor will be incubated for approximately 24-48 hours, or until the monolayers are approximately 50 per cent confluent, at which time inhibitors will be added. The cell yields from each test system will be determined in triplicate at 24 hour intervals through 6 days of incubation and compared with the cell yields from untreated control cultures. Only inhibitors that have no effects on plating efficiency and cell growth will be used in all subsequent carcinogen studies.

made by the method of Sierne and Davis (manuscript submitted for

Preliminary studies in our laboratory have shown that metyrapone, 4-phenylimidazole and 1-benzylimidazole have no adverse effects on the plating efficiency and growth of human embryonic lung cells and epidermoid lung carcinoma cells at concentrations of 1 and 2×10^{-4} M.

- f. Carcinogen effects on in vitro cell cultures

- 1) Plating efficiency and growth rate. The effects of carcinogens on the plating efficiency and growth rate of each cell line will be studied in the presence and absence of cytochrome P-450 inhibitors.

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Details of methodology are described above. Since the carcinogens will be prepared in dimethylsulfoxide (DMSO), both untreated cultures and cultures treated with an equivalent concentration of DMSO will serve as controls. These studies also will include microscopic examination of cover glass cultures after staining with methyl green pyronine and may Grunwald-giemsa for observation of possible variation in cell morphology. Cultures will be cultured in Eagle's MEM containing 10 per cent fetal calf serum.

2) Localization of tritium labeled carcinogens. In addition to analyses of the tritium concentrations in isolated subcellular fractions (described below), the cellular patterns of tritium localization will be observed in autoradiographs of cover glass cultures. Autoradiographs will be prepared using Kodak NTB3 liquid emulsion, exposed, developed and stained as described by Roberts (Am. J. Pathol. 49: 889, 1966).

3) Effects of carcinogens on DNA synthesis in vitro. The influence of the carcinogens on DNA synthesis and generation times in the presence and absence of cytochrome P-450 inhibitors will be determined in each cell line by studies on the incorporation of tritiated thymidine (TdR³H). Using unlabeled carcinogens, the cultures will be pulsed for 4 hours with 0.5 μ Ci per ml of TdR³H at 24 hour intervals through 6 days of incubation. The culture processing, DNA and protein extraction and scintillation counting to measure uptake of TdR³H will be performed as described by Capary and Hughes (J. Immunol. Methods 1: 263, 1972). All analyses will be performed in triplicate cultures as described above. Untreated cultures and DMSO treated cultures will serve as controls.

At each study interval, cover glass cultures will be prepared for autoradiographic determination of the per cent of labeled cells.

The number of labeled cells per culture thus will be compared and correlated with the quantitative assays of TdR³H incorporation by the total cell population.

The generation time will be measured on the biphasic curve representing the percentage labeled metaphases obtained after the pulse labeling of actively growing cells, as described by Quastler and Sherman (Exp. Cell Res. 17: 420, 1959). The periods of exponential growth will be established with untreated control cultures of each cell line to be investigated.

4) Transformation of in vitro cultures. The potential for induction of malignant transformation by the carcinogens in cultures of human embryonic lung and of normal and dwarf mouse lung will be monitored. These studies will require the maintenance of carcinogen-treated cells in continuous cultures for a period of several weeks. Periodic microscopic examination of the cultures will reveal the commonly used criteria for transformation, such as a change in morphology or a change in the social behavior of the transformed cells. These changes include palisading of cells with loss of contact inhibition. These converted cells often form high density colonies which can be seen macroscopically. Colony counts will be performed to obtain a quantitative measure of transformation (Hartley and Rowe, Proc. Nat.

Acad. Sci. 55: 780, 1966).

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The transformed colonies will be isolated by microcloning techniques, as described by Robb (Science, 170: 857, 1970). Cell cultures derived from the cloned colonies will be characterized by studies on growth rate and generation time and by karyology (in Tissue Culture Methods and Applications, edited by P. F. Kruse and M. K. Patterson, Jr., Chapter 15, 1973).

Cell cultures derived from transformed clones will be further characterized for chemical oncogenesis by tests for tumor induction in the proper host. Successful systems reported include the hamster embryo cell system (Berwald and Sachs, J. Nat. Cancer. Inst. 35: 641, 1965), the C₃H mouse prostate system (Chen and Heidelberger, Intern. J. Cancer 4: 166, 1969) and 3T3-like cell lines derived from C₃H mouse embryo cells (DiPaolo, Takano and Popescu, Cancer Res. 32: 2686, 1972). In these systems, the transformed clones give rise to fibrosarcomas on inoculation into the proper host.

The inbred normal and dwarf mouse systems proposed in our studies will offer suitable models in which to test the carcinogen transformed mouse clones for tumorigenesis, thus confirming the malignant properties of the transformed cells.

The growth of human malignant tumor tissue in irradiated, thymectomized mice also has been reported (Castro, Nature New Biol., 239: 83, 1972). In addition, cultured human malignant cell lines have been shown to form progressively growing tumors in thymectomized rats simultaneously treated with antirat lymphocyte serum (Plata et al., J. Natl. Cancer Inst., 50: 849, 1973). We propose, therefore, to test transformed cloned cells from carcinogen-treated human embryonic lung cell lines for tumor induction in immunosuppressed mice.

2. DNA Labeling by Metabolism of Tritiated Carcinogens in Lung Cells Grown in Culture.

- a. We shall determine the amount of tritiated compound bound to the DNA fractions of lung cells grown in culture in the presence and absence of cytochrome P-450 inhibitors in order to study the role of cytochrome P-450 mediated hydroxylations and cytochrome P-450 independent hydroxy-methylation on the activation of carcinogenic polycyclic hydrocarbons delineated above. The tritiated hydrocarbons will be prepared from the parent hydrocarbons by the Wiltzbach tritium exchange technique and/or catalytic exchange labeling to be performed by INC Pharmaceuticals, Inc., 2727 Campus Drive, Irvine, California 92664.

The techniques to be employed in these experiments are those described by Rayman and Dipple (Biochemistry 12: 1538, 1973).

One of us (Nathan Sloane) will be at the Jackson Laboratories, Bar Harbor, Maine during June, July and August, 1974, to study the isolation of DNA fractions by density gradient centrifugation and also to work with the effect of ACTH on the liver RNA/DNA ratio of dwarf mice compared to the congenic litter mates. Dr. Sloane will be a Visiting Investigator sponsored by Dr. Harry Chen.

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3. RNA and Protein Labeling by Metabolism of Tritiated Carcinogens in Lung Cells Grown in Culture

We shall determine the amount of tritiated compound bound to the RNA and protein fractions of cells grown in culture in the presence and absence of cytochrome P-450 inhibitors. The techniques to be employed in these studies will be those described by Duncan and Brookes (Int. J. Cancer 6: 496, 1970).

C. Effects of Benzo(a)pyrene Polycyclic Hydrocarbons and Derivatives on Tissue Culture Cells: A Brief Literature Review.

Recent evidence suggests strongly that the epoxide of polycyclic hydrocarbons, which is formed by a mixed function oxidase in microsomes, is a proximate carcinogen (Sims, Biochem. J. 84: 558, 1962; Boyland and Sins, Biochem. J. 95: 778, 1965; Silkirk, Huberman and Heidelberger, Biochem. Biophys. Res. Commun. 43: 1010, 1971).

Gelboin et al. (Proc. Nat. Acad. Sci. U.S. 64: 1188, 1969) showed a direct correlation between the level of the enzyme benzo(a)pyrene hydroxylase and the susceptibility of the tissue culture cells to cytotoxicity produced after treatment with benzo(a)pyrene; furthermore these investigators showed that 3-hydroxybenzo(a)pyrene was cytotoxic to cells that were resistant to the cytotoxic effect of benzo(a)pyrene per se.

However, Marquardt et al. (Proc. Am. Assoc. for Cancer Res. Abs. 27, 1973) reported that the k region epoxides of 7, 12-dimethylbenz(a)anthracene and 7-hydroxymethylbenz(a)anthracene were more lethal but produced fewer transformed foci than the parent hydrocarbon with mouse prostate fibroblasts. Thus the involvement of epoxides in carcinogenesis by these hydrocarbons remains in doubt.

These latter results appear contradictory to data reported by Gelboin and Wiebel (Science 170: 169, 1970); who showed that 9, 10-dimethylbenzanthracene tumorigenesis was inhibited by 7, 8-benzoflavone, an inhibitor of aryl hydrocarbon hydroxylase. However, Wheatley (Brit. J. Cancer 2: 787, 1968) showed that SKF-525-A (an inhibitor of cytochrome P-450) enhanced mammary tumorigenesis by 7, 12-dimethylbenz(a)anthracene. However, metabolism of certain methyl derivatives involves formation of hydroxymethyl derivatives which may also be proximate carcinogens (Boyland, Sims, Huggins, Nature, 207: 816, 1965; Flesher, Sydnor, Cancer Res. 31: 1951, 1971). Epoxidation of methyl polycyclic hydrocarbons appears minimal (Boyland and Sims, Biochem. J. 95: 780, 1965).

The contradictions implicit in these two mechanisms of carcinogenesis by hydrocarbons have been mitigated by a recent finding of Flesher and Sydnor (Abs. p. 55, No. 217: 63rd Annual Meeting Proc. Am. Assoc. for Cancer Research, May 4-6, 1972, Boston, Massachusetts) who demonstrated the 6-hydroxymethyl derivative as one of the metabolites of benzo(a)pyrene. Thus hydroxymethylation may be the route to form a proximate carcinogen, which is common to polycyclic hydrocarbons. This metabolic pathway should be investigated further.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

The University of Tennessee, Department of Biochemistry, School of Medicine has office, laboratory, library, shop, stockroom and coldroom facilities to carry out the proposed research. The following items are available: Amino acid analyzer, chromatographic equipment, high voltage paper electrophoresis equipment, animal facilities, UV and IR spectrometers, Model E ultracentrifuge, liquid scintillation counting equipment and Craig automatic counter-current distribution apparatus.

Dr. Roberts' research facilities in the Department of Microbiology consist of 265 sq. ft. of office space and 1130 sq. ft. of laboratory space. Adequate facilities are available for autoradiography, fluorescence microscopy, tissue culture, glassware preparation and the housing and care of laboratory animals. The following equipment is also available: Sorvall RC2-B superspeed refrigerated centrifuge with 3 rotors, Isco Fraction Collector, DG fractionator, UV monitor and recorders equipped with flow cells, DB and column fractionation apparatus, Bausch and Lomb refractometer, Nuclear Chicago Scintillation Spectrometer, Gilford Model 240 spectrophotometer, Zeiss bright field and fluorescent microscope, AO bright field microscope, immunoelectrophoresis apparatus, Sorvall GLC-1 centrifuge with 2 rotors, low speed counter-top centrifuge, Mettler microbalance and small laboratory balances, Torsion balance and pH meter, microtissue homogenizer, -20C and -70C freezers, 5C refrigerators, 37 C incubators, waterbaths, Virtis freeze-dry apparatus, Spinco Model L preparative ultracentrifuge, Packard Tri-Carb liquid scintillation spectrometer, Spinco L2B-65 preparative ultracentrifuge and several rotors, preparative gel electrophoresis apparatus, Sonifier, liquid nitrogen cell culture storage tank, and two large CO₂ incubators.

11. Additional facilities required: None

1003545309

12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

14. First year budget:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s)
even if no salary requested)

	% time	Amount
Nathan Sloane, Ph.D.	40	0
Audrey Roberts, Ph.D.	30	0

Technical

Helen Hill, Laboratory assistant (Salary \$5,200; fringe @ 8.5% \$442)	100	5,642
Tissue Culture technician, B.S. (to be recruited) (Salary \$7,200; fringe @ 8.5% \$612)	100	7,812
Chemical research assistant (to be recruited) (Salary \$5,400; fringe @ 8.5% \$459)	100	5,859

Sub-Total for A \$ 19,313

B. Consumable supplies (by major categories)

Tissue culture glassware and plastic disposables	2,400
Tissue culture media and serum	5,000
(One liter of 10X medium and one liter fetal calf serum per wk)	
Biochemical supplies	2,000
Vivarium charges for animal care and animal costs	1,000

Sub-Total for B 10,400

C. Other expenses (itemize)

Travel (to attend scientific meetings)

Sub-Total for C 800Running Total of A + B + C 30,513

D. Permanent equipment (itemize)

None

Sub-Total for D 0E 4,577

E. Indirect costs (15% of A+B+C)

Total request 35,090

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	20,283	10,500	800	0	4,738	36,321
Year 3	21,297	10,500	800	0	4,890	37,487

1003545310

16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Ring Hydroxymethylation in Hydrocarbon Carcinogenesis	American Cancer Society Grant BC 137	\$26,000	July 1, 1973- June 30 1975

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigators Nathan H. Sloane, Ph.D.
Audrey N. Roberts, Ph.D.

Typed Name Nathan H. Sloane
Signature Audrey N. Roberts Date 1/24/74
Telephone 901-528-6160
Area Code Number Extension

Checks payable to

Mr. C. Q. Tipton, Vice Chancellor for Business & Finance

Mailing address for checks

800 Madison Avenue

Memphis, Tennessee 38163

Responsible officer of institution

Typed Name Edmund Pellegrino
Title Chancellor
Signature Edmund Pellegrino Date 1-25-74
Telephone (901) 528-5512
Area Code Number Extension

1003545311

#979 - TRIOLO

1003545312

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

February 11, 1974

Grant application No. 979

CANCER

To: The committee comprising Drs. Gardner, Huebner, Meier

Subject: Anthony J. Triolo, Ph.D., Jefferson Medical College,
Thomas Jefferson University, Philadelphia
New application No. 979
"Nicotine on the Metabolism and Carcinogenesis of
Benzo(a)pyrene"

History

A preliminary inquiry was handled as Case No. 259,
and formal application was encouraged.

Request

Application No. 979 requests \$48,493, plus two additional years.

Document Submitted

Enclosed is application dated Jan. 21, 1974 (16 pages plus appendix).

F.W.N.
F.W.N.

FWN:wg
Encl.

1003545313

THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

FEB 5 1974

Date: Jan. 21, 1974

1. Principal Investigator (give title and degrees):

Anthony J. Triolo, B.Sc., M.Sc., Ph.D.
Associate Professor of Pharmacology

2. Institution & address:

Jefferson Medical College
Thomas Jefferson University
1025 Walnut Street
Philadelphia, Pa. 19107

3. Department(s) where research will be done or collaboration provided:

Departments of Pharmacology & Pathology
1020 Locust Street
Philadelphia, Pa. 19107
Telephone: 215-829-7974

4. Short title of study:

Nicotine on the metabolism and carcinogenesis of benzo(a)pyrene

5. Proposed starting date:

July 1, 1974

6. Estimated time to complete: Approximately 2 years for the nicotine and benzo(a)pyrene studies

7. Brief description of specific research aims:

As part of a collaborative project with the Department of Pathology, this study has the goal of determining whether exposure to low levels of nicotine affects the metabolism and carcinogenicity of benzo(a)pyrene (BP). A/He J mice will be used, since this strain is highly susceptible to BP induced pulmonary and stomach tumors. Some of the fundamental questions that we will attempt to answer as part of our investigation include:

(1) Does chronic feeding for 90 days of nicotine alter the activity of the enzyme BP hydroxylase in lung and stomach and thus influence the tumor incidence at these target organs resulting from the administration of BP?

(2) Can the dietary feeding of nicotine affect the in vitro and in vivo rate of metabolism of BP to form the ultimate carcinogen BP-epoxide?

(3) Are the levels of dietary intake of nicotine and the resulting blood levels of nicotine, as it may enter into the above types of interactions with BP, realistic in terms of the human blood levels of nicotine that are encountered in cigarette smokers?

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2.
8. Brief statement of working hypothesis:

A variety of chemical agents have been shown experimentally to alter the activity of the microsomal enzyme, aryl hydrocarbon hydroxylase (BP-hydroxylase), and thus influence the carcinogenicity of several polycyclic aromatic hydrocarbons (PAH) such as BP. Information is limited, however, on the influence of exposure to nicotine on the metabolism and carcinogenic action of these PAH. We have shown that acute administration of nicotine to rats inhibited the in vivo and in vitro metabolism of BP. The suggested hypothesis of the carcinogenic action of PAH involves their metabolic activation by microsomal enzymes to reactive intermediate epoxides that bind readily to DNA of target organs. Thus, an important aspect of carcinogenesis is the formation of a reactive epoxide, the level of which might be influenced by other chemical agents such as nicotine through its effect on microsomal enzyme activity required for the metabolism of PAH. The accumulation of evidence that prolonged heavy cigarette smoking in humans plays a role in the etiology of cancer of the lung emphasizes the need for more extensive studies of the metabolic and carcinogenic interactions between BP, a known carcinogen, and nicotine, both of which are present in tobacco smoke.

9. Details of experimental design and procedures (append extra pages as necessary)

Experiments will be conducted with female A/He J mice at 9-10 weeks of age obtained from the Jackson Laboratory, Bar Harbor, Maine. Animals will be randomized by weight and placed on experimental diets of Purina Rat Chow to which has been added various levels of nicotine. An illustration of the procedural approach we plan to use in testing the interaction of nicotine on the tumor incidence of BP is as follows: The maximum amount of nicotine in the diet that does not effect the growth rate of rats was reported as 60 ppm (Wilson and De Eds, 1936). Since data are not available to show whether mice will tolerate this level of nicotine, a preliminary pilot study will be conducted in small groups of animals (5 each) to determine the maximum level of nicotine in the diet that does not inhibit growth rate. This will be accomplished by feeding mice for 30 days nicotine at 2-fold increments (15.0, 30.0, 60.0 and 120 ppm) above and below the maximum tolerated dose for rats. Subsequently, mice in groups of 20 each will be fed for 90 days three graded levels of nicotine up to the maximum dose tolerated for 30 days. Corn oil used as a vehicle for the addition of nicotine to the experimental diets will be incorporated in the diet of the control animals.

In accord with the procedure described by Wattenberg (1973), the induction of pulmonary adenoma will be accomplished by administering 3 mg of BP orally on the 4th and 18th day after starting the experimental diet. Forestomach tumors of the mouse will be induced by addition of BP (1000 ppm) in the diet during a 90 day feeding period as reported by Wattenberg (1972). Animals receiving various dietary levels of nicotine alone, and a separate group receiving corn oil in Purina Rat Chow, will serve as controls and both groups will be examined for spontaneous pulmonary and gastric tumors after the termination of the experimental feeding. The following list shows the dietary components of the various groups used in the 90-day feeding study:

- #1 Purina chow + corn oil
- #2 Purina chow + corn oil + nicotine (x) ppm
- #3 Purina chow + corn oil + nicotine (2x) ppm
- #4 Purina chow + corn oil + nicotine (4x) ppm
- #5 Purina chow + corn oil + nicotine (x) ppm + BP
- #6 Purina chow + corn oil + nicotine (2x) ppm + BP
- #7 Purina chow + corn oil + nicotine (4x) ppm + BP

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Food consumption, nicotine intake and body weight will be monitored weekly during the total period of the experiment, after which the mice will be

autopsied and their lungs and stomach studied. The number and histologic type of tumors in lung and stomach will be determined, and differences in tumor incidence between control and experimental groups will be expressed by the Carcinogenic Index (the percent of mice with tumors times the mean number of tumors in positive animals).

After termination of the experimental feeding, the in vitro metabolism of BP (e.g., BP-epoxide and hydroxylated products) by liver homogenates from controls (corn oil in Purina Rat Chow) and from animals placed on nicotine diets alone, will be determined by the methods described below. Similarly, blood samples will be obtained before autopsy from all animals on the experimental diet containing nicotine alone, and from animals receiving the diet containing nicotine plus BP, for the analysis of nicotine blood levels by the gas-chromatographic procedure described below.

An attempt will be made to show a correlation between the tissue levels of formed BP-epoxide and any changes observed in the incidence of BP induced tumors. For this purpose, a separate group of feeding experiments will be run to determine the relationship between the dose of nicotine and the in vitro and in vivo formation of BP-epoxide derived from BP by the methods described below.

In vitro Metabolism of BP

The activity of BP hydroxylase will be determined in various tissues. The method to be used involves the measurement of fluorescence in an Aminco-Bowman spectrophotofluorometer of hydroxylated metabolites of BP by the general procedure of Dehnen et al. (1973). Since a problem may exist in determining low levels of BP hydroxylase activity in some tissues (e.g. forestomach), the method described by Dehnen et al. (1973) was selected because it is more sensitive for this assay and extraction of metabolites with organic solvents is not required as in other available methods. The determination of BP hydroxylase activity in tissue with high activity, such as liver, is also important, because changes in enzymatic activity at this site by exposure to nicotine and BP may alter the tissue levels of BP and its metabolites in target organs (e.g. lung and forestomach) that are susceptible to BP induced tumors. Enzyme activity will be expressed as the quantity of reference standard 3-hydroxy-BP causing fluorescence equivalent to total hydroxylated metabolites produced per mg of tissue during the time of incubation. Optimal conditions for all enzyme reactions will be determined by testing various incubation times and enzyme and substrate concentrations.

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For the analysis of BP-epoxide formed in vitro by incubating tissue homogenates with BP, the method used will be that of Grover et al. (1972). This procedure requires the incubation of liver microsomes equivalent to 10 gm liver with cofactors required for an NADPH-generating system, and pre-incubating (30°C for 10 min) with 1,2 epoxy-1,2,3,4-tetrahydronaphthalene, to increase the yield of epoxides by inhibiting the enzyme-catalyzed (by epoxide hydrase) conversion of epoxides to dihydrodiols. The reaction is initiated by the addition of tritiated BP (400 ugm), and after incubation the residue of an ether extract is chromatographed on an alumina column and partitioned into fractions of BP, BP-epoxide and hydroxylated products. Though application of this method to tissue with low enzyme activity (e.g. lung and forestomach) may be difficult, an attempt will be

made to determine the in vitro metabolism of BP to its epoxide in these tissues from controls and animals receiving various dietary levels of nicotine. In similar experiments an attempt will also be made to measure the tissue level of BP-epoxide in vivo derived from the intravenous administration of BP either during the dietary feeding of nicotine or shortly after the 90-day feeding period.

Blood Levels of Nicotine

Animals will be bled by cervical incision after dietary feeding of nicotine and the blood will be collected in heparinized tubes. Nicotine will be analyzed according to the procedure described by Isaac and Rand, 1972. After centrifugation of blood the internal standard modaline is added to plasma which is then alkalized and extracted with diethyl ether. The ether extract is reduced to an appropriate volume and 2.0 ul are injected into the gas chromatograph. A Hewlett-Packard model 5750B gas chromatograph fitted with an alkaline flame ionization detector will be used to analyze plasma extracts of nicotine.

Statistics

Only probability values of 5% or less will be considered significant for all data obtained. All experiments will be designed so that the results may be subjected to statistical analysis using, when appropriate, student t-test and coefficient of correlation.

References

1. Dehnen, W., Tomingas, R. and Roos, J. A modified method for the assay of benzo(a)pyrene hydroxylase. *Anal. Biochem.* 53: 373, 1973.
2. Grover, P.L., Hower, A. and Sims, P. Formation of K-region epoxides as microsomal metabolites of pyrene and benzo(a)pyrene. *Biochem. Pharmacol.* 21: 2713, 1972.
3. Isaac, P.F. and Rand, M.J. Cigarette smoking and plasma levels of nicotine. *Nature* 236: 308, 1972.
4. Wattenberg, L.W. Inhibition of carcinogenic and toxic effects of polycyclic hydrocarbons by phenolic anti-oxidants and ethoxyquin. *J. Natl. Cancer Inst.* 48: 1425, 1972.
5. Wattenberg, L.W. Inhibition of chemical carcinogen-induced pulmonary neoplasia by butylated hydroxyanisole. *J. Natl. Cancer Inst.* 50: 1541, 1973.
6. Wilson, R.H. and DeEds, F. Chronic nicotine toxicity: I. Feeding of nicotine sulfate, tannate and bentonite. *J. Indust. Hyg. Toxicol.* 18: 553, 1936.

1003545317

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Facilities for this project include three fully-furnished research laboratories throughout the year and an adjacent student laboratory for nine months of the year. Adequate animal rooms and caging are available within the department, and we also have use of an institutional central animal quarters. Workers on this project have access to departmental stocks of standard chemicals, drugs, glassware, apparatus and instruments for routine toxicologic and pharmacologic experimentation.

Equipment now located in the three "central base" laboratories include: 2 fume hoods, 1 Labline incubator, 2 Eberbach water bath shakers, 1 torsion and 2 roller Smith Precision Balances for tissue weighing, 2 pH meters, 2 high speed refrigerated centrifuges (International and Sorvall), 1 F&M Gas Chromatograph with flame ionization, 1 Hewlett-Packard Gas Chromatograph, flame ionization and electron capture detectors, 4 Vortex mixers, 2 hot air drying ovens, 3 hot plates, 3 timers, 1 Gilford spectrophotometer, and 2 Bausch and Lomb Spectronic 20 colorimeters. Specialized equipment located elsewhere in the department but available for this project include cold rooms (refrigerator and freezer), incubator room, 2 Spinco Model L ultracentrifuges, 1 Perkin Elmer infrared spectrophotometer, 2 Packard liquid scintillation spectrometers, 1 Aminco-Bowman spectrophotofluorometer, and 1 Aminco Chance Dual Wavelength/Split Beam Spectrophotometer.

11. Additional facilities required: None

12. Biographical sketches of investigator(s) and other professional personnel (append):

Pages 6 through 15 of grant proposal

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).
(See page 3-A)

1003545318

3-A

13. A. Manuscript accepted in Science pertinent to this grant proposal is appended to application (page 16).

B. Five (5) publications appended to application (page 17).

1003545319

Salaries (give names or state "to be recruited")
Professional (give % time of investigator(s)
even if no salary requested)

	% time	Amount
Anthony J. Triolo (Princip. Invest.)	30	\$ 6,270
Julius M. Coon (Co-Princip. Invest.)	15	5,760
Gonzalo E. Aponte (Co-Princip. Invest.)	5	3,000
Post-Doctorate (to be recruited)	100	11,000

Technical

Research Technician (to be recruited)	50	4,000.
Fringe benefits on above (13%)		3,903

Sub-Total for A 33,933

B. Consumable supplies (by major categories)

Animal, animal care	2,500
Prepared purina diets	1,000
Chemicals & cofactors	1,500
Glassware & misc.	1,000

Sub-Total for B 6,000

C. Other expenses (itemize)

Equipment maintenance & repair	800
Laundry	200
Travel to scientific meetings	600

Sub-Total for C 1,600

Running Total of A + B + C 41,533

D. Permanent equipment (itemize)

High sensitivity nitrogen detector
Model #1516B Hewlett-Packard
for plasma analysis of nicotine

Sub-Total for D 730

E. Indirect costs (15% of A+B+C)

E 6,230

15. Estimated future requirements:

Total request 48,493

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	\$35,968	\$6,300	\$1,600	--	\$6,580	\$50,448
Year 3	30,484	6,615	1,600	--	5,805	44,504

1003545320

16. Other sources of financial support: None

List financial support from all sources, including own institution, for this and related research projects.

Approximately \$1000 available from the Department of Pharmacology operating budget

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
	None		

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Organophosphates on benzo(a)pyrene carcinogenesis	National Institute of Health Submitted Jan., 1974	\$179,744	Sept. 1, 1974 to Aug. 31, 1979

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Anthony J. Triolo, Ph.D.

Signature Anthony J. Triolo Date Jan. 22, 1974

Telephone 215 829 7974
Area Code Number Extension

Responsible officer of institution

Typed Name William F. Kellow, M.D.

Title Dean Vice President

Signature William F. Kellow 1/28/74

Telephone 215 829 6980
Area Code Number Extension

* Checks payable to

Jefferson Medical College
Thomas Jefferson University

Mailing address for checks

Mr. Frank J. O'Brien, 1020 Walnut St.,

Philadelphia, Pa. 19107

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EPIDEMIOLOGY

1003545322

#826A - DAWBER

1003545323

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

February 12, 1974

Grant application No. 826A

EPIDEMIOLOGY

To: The committee comprising Drs. Gardner, Sommers and Lynch

Subject: Thomas R. Dawber, M.D., M.P.H., Boston Univ. School of Medicine
Continuation application No. 826A
"Epidemiologic Study of Cigarette Smoking and Cardiovascular Disease"

History

This study has been supported by CTR since 1971. The current grant, in the amount of \$54,625, is for the final year of a three-year program.

Therefore the enclosed request competes without commitment.

Request

Application No. 826A requests \$54,625 plus one additional year.

Document Submitted

Enclosed is application dated Jan. 24, 1974 with appendices A, B and C.

Comment

Dr. Gardner has requested evaluation of this proposal from Dr. Seltzer.

FWN:gh

Encls.

FWN
F.W.N.

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ad
1/21/74
sh

THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.

110 EAST 50TH STREET

NEW YORK, N. Y. 10022

(212) 421-8985

Application for Research Grant

(Use extra pages as needed)

JAN 31 1974

Date: Jan. 24,
1974

1. Principal Investigator (give title and degrees):

Thomas R. Dawber, M.D., M.P.H.

H. Emerson Thomas, Jr., M.D.

Carl C. Seltzer, Ph.D.

Principal Investigator

Co-Investigator

Co-Investigator

2. Institution & address:

Boston University School of Medicine

80 East Concord Street

Boston, Ma. 02118

3. Department(s) where research will be done or collaboration provided:

Department of Medicine

Section on Preventive Medicine & Epidemiology

Evans Department of Medical Research

4. Short title of study:

Epidemiologic Study of Cigarette Smoking and Cardiovascular
Disease

5. Proposed starting date: July 1, 1974

6. Estimated time to complete: Two years

7. Brief description of specific research aims:

To evaluate the effect of age on the relationship between
cigarette smoking and coronary heart disease, stroke and
peripheral vascular disease.

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8. Brief statement of working hypothesis:

2.

The reported effect of cigarette smoking on the development of coronary heart disease, stroke and peripheral vascular disease is not one related to atherogenesis but to other factors which affect the development of an overt episode, e.g. thrombosis or arrhythmia. Persons susceptible to these events develop them earlier if they smoke cigarettes. The net effect is to cause clinically overt disease to appear earlier in cigarette smokers than non-smokers. Ex-smokers will be at an even lower risk than those who never smoked. A third observation should be the disappearance of the effect of cigarette smoking with increasing age as susceptible persons are removed from the population.

If the above hypotheses are correct, our approach to the cigarette smoking risk insofar as coronary heart disease is concerned is development of practicable methods of detecting highly susceptible persons as early in life as possible.

9. Details of experimental design and procedures (append extra pages as necessary)

The proposed study is a continuation of existing investigations of risk factors and cardiovascular disease in the Framingham Study. In July 1971 a grant was awarded to the Boston University Medical Center by the Council for Tobacco Research to assist in the continuation of the Framingham Study. This longitudinal investigation of the epidemiology of coronary heart disease was begun in 1949 - 1950 by the National Heart and Lung Institute and has followed the original cohort population of 5,209 subjects selected in January 1950. This population aged 30 - 59 at the onset of the study is now 53 - 82. The population has decreased through death to 4,076 subjects as of Nov. 30, 1973.* At the time the NHLI first decided to phase out the study it was unclear exactly what was planned. Later when the Boston University Medical Center actively developed a program aimed at continuing the Study the NHLI decided to maintain surveillance of causes of death and to follow-up on such hospitalizations as were possible. Readily available was information from the local Framingham Union Hospital where the study was located. Information from other hospitals, however, usually depended on knowledge of an illness which could only be obtained from direct contact with the patient or an immediate relative.

In view of the NHLI decision to maintain a limited population surveillance in Framingham the task of the BUMC - Framingham Study was somewhat lessened, but the big task of direct examination of the available members of the cohort required a major effort. The BUMC - Framingham Study began in July 1971 to plan the twelfth re-examination of the population. This required development of new examination forms, recruitment of a staff to bring in subjects for examination, conduct the examinations, process the data, etc. All this was rapidly

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*see Appendix A

9. Details of experimental design and procedures (cont.)

accomplished and the Study was reactivated in September 1971. During the next two years the available population was re-examined for the twelfth time. This examination has been completed as of January 1, 1974.*

Meanwhile, Examination 13 was begun in September 1973, again to be conducted over a two-year period. Processing of the data collected at Exam 12 has been on-going during the examination period.

The availability from the NHLI of data previously collected in Framingham has been delayed by actions of that organization. This was done on the grounds that until the present BUMC - Framingham Study investigators had completed the examination of the cohort they did not have any need for previous data which belonged to the NHLI and the analysis of which was not a proper function of others than the NHLI investigators. This delay has made it impossible for the present investigators to carry out certain preliminary studies which they proposed in the previous grant application until past data could be acquired.

In view of the NHLI attitude the principal investigator sought certain data on his own initiative and has been able to put together sufficient information from previous examinations to make possible the analysis of data on smoking in relation to a number of disease end points and also to other factors of risk should the NHLI not see fit to provide the desired information.

Tapes covering data from the previous examinations at Framingham have now been received and constitute a better set than other data available. These data are for our use in analyzing data collected by us in the twelfth and subsequent examinations. Publication of reports covering the entire data is understood to be our prerogative.

In this regard the nature of the relationship between the current operation conducted by the NHLI in Framingham and the Boston University Medical Center - Framingham Study should be made clear. The NHLI is currently funding a follow-up of death and hospitalization of subjects in the Study while our activity involves a clinical appraisal of all living subjects. We also participate in the review and collection of the pertinent data in which the NHLI has a legitimate interest. We collaborate with the NHLI staff in conducting the entire study. This has included a number of scientific publications. Because the data in these

*See appendix B and C

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9. Details of experimental design and procedures (cont.)

has essentially all come from previous data collected by the NHLI such publications have needed approval from that organization.

Now that we have completed the twelfth examination and have received tapes on the past data a larger number of publications will result directly from our own group. Such reports will represent the opinions of our own investigators and not be subject to NHLI review. At the same time, publications will continue to be generated by the NHLI staff and, if involving data obtained by us, will be published as a joint project.

We currently have made a review of the number of disease endpoints which have been accumulated and will now be able to provide the investigators with data for the study of smoking in relation to coronary heart disease, stroke and peripheral arterial disease in twenty-two years of observation. This will enable us to determine what changes have taken place, if any, in the risk of those manifestations of cardiovascular disease with changes in age. Dr. Seltzer plans a study of the data pertaining to the predisposition of ^{smoking habits and} alcohol consumption and development of coronary heart disease. Data which he wishes should be in his hands sometime in February 1974.

One approach has been to determine the "immediate" risk of cigarette smoking over an arbitrarily selected period of time following the observations of smoking habits. This risk is determined for each age bracket. Current observations have been confined to myocardial infarction but steps have been taken to widen these to include the other manifestations of coronary disease, stroke and peripheral arterial disease.

In order to carry out this study it is necessary to know the current smoking status of all the subjects since this status is constantly changing. To obtain such information requires the kind of direct continued surveillance of the Framingham Study population which has been made possible by the Boston University Medical Center effort and for which funds have been contributed by foundations, the insurance industry, pharmaceutical firms and large numbers of private individuals, both participants and non-participants as well as by grant support from the N.I.H. and the Council for Tobacco Research.

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9. Details of experimental design and procedures (cont.)

From the data so obtained it then becomes possible to evaluate the "immediate" risk on the basis of known smoking status rather than on an assumption of unchanged smoking habits which has characterized reports from Framingham, i.e. based on the reported habits at the initial examination.

Preliminary observations (on which the attached abstract submitted for presentation of a paper on the subject at the next American College of Physicians meeting was based) indicate clearly that there is a decrease in risk of cigarette smoking insofar as myocardial infarction is concerned with increasing age beginning with the youngest subjects studied. This risk may be partially due to the failure to fully account for all subjects who have discontinued smoking but future analysis will take this into consideration. As the subjects reach the age of 60 - 70 the effect of cigarette smoking on myocardial infarction disappears and the trend suggests strongly that a reversal of the effect with a lesser risk in the smokers than in the non-smokers above this age.

Because of the relatively small number of older subjects there is a need for follow-up of the Framingham population for a few more years (two may be sufficient) to enable the suggested findings to be adequately confirmed.

The data from Exam 12 can now be added to the data from previous examinations and a number of reports can now be prepared. A grant for five years from the National Institute for Neurology and Stroke was provided in 1971. The present grant request is therefore made for a two-year period to run concurrently with the NIH grant to enable the BUMC - Framingham Study investigators to complete the task of examining the study population, analyzing data from the 12th and 13th examinations as well as from previously collected data and preparing the desired reports. The next two years will be utilized in reporting the findings from a total of 22 years of observation as well as continuing further study of the population.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Space and facilities available to the National Heart and Lung Institute in Framingham under an agreement with the Framingham Union Hospital have been utilized by the Boston University Medical Center - Framingham Study at no cost. It is anticipated that this agreement will continue but with some modification requiring the BUMC - Framingham Study to acquire some additional space by direct negotiation with the Framingham Union Hospital.

11. Additional facilities required:

Additional space requirements were anticipated last year due to a projected change in space utilization by the Framingham Union Hospital. That change has not as yet materialized but will certainly do so during the current year necessitating rental of the additional space during the coming year.

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12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

14. First year budget:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s)
even if no salary requested)

	% time	Amount
Thomas R. Dawber, M.D., M.P.H.	40%	\$12,000
H. Emerson Thomas, Jr., M.D.	25%	6,000
Carl C. Seltzer, Ph.D.	20%	5,000
Philip A. Wolf, M. D.	--	--

Technical

Clerk typist	100%	8,250
Statistical clerk	100%	8,250

Sub-Total for A 39,500

B. Consumable supplies (by major categories)

Record forms	500
Office supplies	500

Sub-Total for B 1,000

C. Other expenses (itemize)

Travel	1,500
Data processing	4,000
Space (40% of cost)	1,500

Sub-Total for C 7,000

Running Total of A + B + C 47,500

D. Permanent equipment (itemize)

Sub-Total for D --

E. Indirect costs (15% of A+B+C)

E	7,125
Total request:	54,625

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	41,000	500	7,000	0.0	8,775	57,275
Year 3						

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BIBLIOGRAPHY

Kannel, W. B. and Dawber, T. R.: The prudent management of the coronary-prone subject; coping with risk factors. In Russek, H.I. and Zohman, B.L. (eds.): Cardiovascular Therapy: the Art and the Science. Baltimore, Williams & Wilkins, 1971, pp. 225-236.

Dawber, T. R. and Kannel, W. B.: Current status of coronary prevention: lessons from the Framingham Study. *Prevent. Med.* 1: 499-512, Dec. 1972.

Kannel, W. B., and Dawber, T. R.: Atherosclerosis as a pediatric problem. *J. Pediat.* 80:544-554, Apr. 1972.

Kannel, W. B. and Dawber, T. R.: Contributors to coronary risk implications for prevention and public health: the Framingham Study. *Heart and Lung* 1:797-810, Nov.-Dec. 1972.

Kannel, W. B., Gordon, T., Wolf, P. A., and McNamara, P.: Hemoglobin and the risk of cerebral infarction: the Framingham Study. *Stroke* 3:409-420, July-Aug. 1972.

Dawber, T. R., Thomas, H. E., Jr., and McNamara, P. M.: Characteristics of the dicrotic notch of the arterial pulse wave in coronary heart disease. *Angiology* 24:244-255, Apr. 1973.

Wolf, P. A., Kannel, W. B., McNamara, P. M., and Gordon, T.: The role of impaired cardiac function in atherothrombotic brain infarction: the Framingham Study. *Am. J. Pub. Health* 63:52-58, Jan. 1973.

Kannel, W. B., and Dawber, T. R.: Hypertensive cardiovascular disease: the Framingham Study. In Onesti, G., Kim, K.E., and Moyer, J. H. (Eds.): Hypertension: Mechanisms and Management. New York, Grune & Stratton, 1973, pp. 93-110.

Margolis, J. R., Kannel, W. B., Feinleib, M., Dawber, T. R., and McNamara, P. M.: Clinical features of unrecognized myocardial infarction -- silent and symptomatic. Eighteen year follow-up the Framingham Study. *Am. J. Cardiol.* 32:1-7, July 1973.

Wolf, P. A., Dawber, T. R., Kannel, W. B., and Gordon, T.: Epidemiologic Assessment of the Stroke Candidate: the Framingham Study. *Neurology* 23:418, 1973 (Abstract)

Wolf, P. A., Kannel, W. B., McNamara, P. M., and Dawber, T. R.: The natural history of stroke: the Framingham Study. *Circulation* 48:49, 1973 (Abstract)

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Gresham, G. E., Fitzpatrick, T., Wolf, P. A., McNamara, P. M., Kannel, W. B., and Dawber, T. R.: The disability status of survivors of completed stroke in the Framingham Study. Circulation 48:49, 1973 (Abstract)

Kannel, W. B., Wolf, P. A., and Dawber, T. R.: Current status of the epidemiology of atherothrombotic brain infarction: 1973 The Milbank Memorial Fund Quarterly, 1974 (In press).

Wolf, P. A.: Hypertension as a risk factor for stroke; proceedings of the ninth Princeton Conference on Cerebral Vascular Diseases, Princeton, N. J., Jan. 1974 (In press).

In preparation

Wolf, P. A., Kannel, W. B., Gordon, T., and Dawber, T. R.: The Stroke Profile: Epidemiologic Assessment of Risk of Stroke.

Wolf, P. A., Kannel, W. B., McNamara, P. M., and Dawber, T. R.: The natural history of stroke: the Framingham Study.

Wolf, P. A., McNamara, P. M., Gordon, T., Kannel, W. B., and Dawber, T. R.: Diabetes as a risk factor in stroke: the Framingham Study

Wolf, P. A., Kannel, W. B., McNamara, P. M., and Dawber, T. R.: Epidemiologic assessment of cigarette smoking in atherothrombotic brain infarction: the Framingham Study. Submitted for presentation to the American Academy of Neurology Meeting, San Francisco, Calif., April, 1974.

Dawber, T. R., et al: Coffee and Coronary Heart Disease. To be presented at the American College of Cardiology meeting, February 12, 1974.

Dawber, T. R., et al: Cigarette Smoking and Age in the Development of Myocardial Infarction - submitted to the American College of Physicians

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Bibliography (cont.)

Presentations:

Wolf, P. A.: The epidemiology of stroke -- tutorial at Harvard School of Public Health - March 1972

Wolf, P. A.: Profile of the potential stroke candidate: assessment of risk. Presented at the American Heart Symposium on Risk Factor Detection and Management, Dallas, Texas. (Nov. 1972)

Wolf, P. A.: Methods in chronic disease epidemiology: stroke. Presented to the Dept. of Epidemiology, McMaster Univ., Hamilton, Ontario - April 1973.

Wolf, P. A.: Epidemiological assessment of the stroke candidate: the Framingham Study. Presented at the 25th annual meeting of American Academy of Neurology, Boston, Ma. (April 1973)

Wolf, P. A.: Epidemiology of Stroke: the Framingham Study. Presented at Union Hospital, Fall River, Ma. (May 1973)

Wolf, P. A.: Rehabilitation in the brain damaged patient. Presented to the American Society of Law & Medicine, Inc., Boston, Ma. Sept. 1973

Wolf, P. A.: The natural history of stroke: the Framingham Study. Presented at the American Heart Association, Scientific Sessions, Atlantic City, N. J. (Nov. 1973)

Wolf, P. A.: Hypertension as a risk factor for stroke. Presented at the Ninth Princeton Conference on Cerebral Vascular Diseases, Princeton, N. J. (Jan. 1974)

Dawber, T. R.: Epidemiology and Natural History of Hypertension Presented at the annual meeting of the Georgia Heart Association in Atlanta, Georgia - September 1973.

Dawber, T. R.: Pooling Data -- Problems and Promises. Presented at a meeting of the American Gerontological Society, Miami, Florida, November 1973

Dawber, T. R.: "Framingham, Tecumseh, and Evans County" Presented at a meeting of the American College Health Association in April 1973.

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Projects for coming year

1. Use of vasculography in determining risk of coronary heart disease incidence
2. Cigar and pipe smoking vs. coronary heart disease risk
3. The effect of age in determining risk of coronary heart disease manifestations including myocardial infarction, sudden death and angina pectoris.
4. The relationship of cigarette smoking to risk of cerebrovascular accidents.

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BOSTON UNIVERSITY MEDICAL CENTER -- FRAMINGHAM STUDY

Status of Exam 12

Cohort available for Exam 12	4,200
Subjects Examined	3,287
Subjects not examined	913
Deaths before exam (prior to 9/73)	124
Living subjects not examined . . .	789
Incapacitated, living in Fram.*	76
Temporary refusal	37
Refusal*	214
Living out of Framingham area . .	103
No response to card mailed* . . .	160
Whereabouts unknown*	151
No information	48

*4 additional deaths occurred after 9/1/73
1 from each category starred

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BOSTON UNIVERSITY MEDICAL CENTER -- FRAMINGHAM STUDY

CEREBROVASCULAR DISEASE REVIEW TO DATE (12/28/73)

Exam	ABI		C.E.		S.H.		I.H.		Other		T.I.A. only		Grand Total	
	M.	W.	M.	W.	M.	W.	M.	W.	M.	W.	M.	W.	M.	W.
1-11	66	79	17	17	10	13	6	5	4	3	9	8	112	125
													237	
12	$\frac{11}{77}$	$\frac{11}{90}$	$\frac{1}{18}$	$\frac{2}{19}$	$\frac{1}{11}$	$\frac{5}{18}$	$\frac{3}{9}$	$\frac{-}{5}$	$\frac{-}{4}$	$\frac{-}{3}$	$\frac{4}{13}$	$\frac{4}{12}$	$\frac{20}{132}$	$\frac{22}{147}$
													279	
13	6	1	4	-	1	-	-	-	-	-	1	1		

ABI = Atherothrombotic brain infarct
 C.E. = Cerebral embolus
 S.H. = Subarachnoid hemorrhage
 I.H. = Intracerebral hemorrhage
 T.I.A. = Transient ischemic attack

(M = men
 W = women)

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INCIDENCE OF CORONARY HEART DISEASE

Exams 2 through 12

	<u>Exams 1 - 9</u>	<u>Exams 10 - 12</u>	<u>Total</u>
Coronary Heart Disease	580	342	922
Coronary Heart Disease other than Angina Pectoris	394	Not available	--
Angina Pectoris	347	188	535
Angina Pectoris, uncomplicated	231	Not available	--
Coronary Insufficiency plus ECG	85	48	133
Myocardial Infarction (ECG)	226	130	356
Myocardial Infarction History plus Transaminase	22	28	50
Coronary Heart Disease (Sudden Death)	84	51	135
Coronary Heart Disease (not sudden death)	88	86	174

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#968 - FRIBERG

1003545340

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

February 13, 1974

Grant application No. 968

EPIDEMIOLOGY

To: The committee comprising Drs. Gardner, Sommers, Lynch

Subject: Lars Friberg, M.D., Karolinska Inst., Sweden
New application No. 968

"The International Collaborative Twin Registry"

History

CTR sponsored planning meeting on International Collaborative Twin Studies at Miami, December 10 - 14, 1973. Seven of the participants (from New Zealand, United States, Belgium, Sweden, Australia, Japan and Finland) were invited to submit proposals. To date five have been received.

Request

For the first year \$15,000 is requested, plus two additional years.

Document Submitted

Enclosed is application dated January 30, 1974, plus Appendix.

Comment

Dr. Carl Seltzer has been asked to evaluate these proposals.

JWM
F.W.N.

FWN:wg
Encl.

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7-966

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

FEB 1974
Date:

January 30, 1974

1. Principal Investigator (give title and degrees):

Lars Friberg, M.D., Professor and Chairman

2. Institution & address:

Department of Environmental Hygiene, The Karolinska Institute,
S-104 01 Stockholm 60, Sweden.

3. Department(s) where research will be done or collaboration provided:

Department of Environmental Hygiene

4. Short title of study:

The International Collaborative Twin Registry

5. Proposed starting date:

As soon as possible

6. Estimated time to complete:

Probably a period of 3 years

7. Brief description of specific research aims:

In a report of an international symposium in San Juan, Puerto Rico, 1969, concerning twin registries in the study of chronic disease with particular reference to the relation of smoking with cardiovascular and pulmonary disease, it was among other things stated that "Twin studies should be extended in a variety of populations in different geographical and ethnical settings" and that "New twin registries suitable for epidemiological studies, should be established".

In order to implement the above recommendations, investigators already active in twin research or interested in establishing new registries, as well as a number of consultants, were invited to a meeting in December, 1973, by the Karolinska Institute and the Council for Tobacco Research - USA, Inc.

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It was decided at the meeting to constitute an international collaborative twin registry. The intent of this enterprise was to form a collaborative group for the coordination of international research on twins. It was also decided that the chairman of the group should be professor Lars Friberg and that he should ask for funds to enable the committee to perform its functions, including arranging meetings, consultations, coordination of activities of participating groups, establishment of the registry, analyzing the findings and preparing applications.

8. Brief statement of working hypothesis:

2.

Not applicable

9. Details of experimental design and procedures (append extra pages as necessary)

See appended notes on the "Organization and coordination of collaborative twin research."

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

The Chairman and the Secretary of the Committee will use the space and facilities available at the Department of Environmental Hygiene of the Karolinska Institute.

11. Additional facilities required:

None

12. Biographical sketches of investigator(s) and other professional personnel (append):

The investigators are known to the Council for Tobacco Research-USA, Inc.

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

Not applicable

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14. First year budget:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s)
even if no salary requested)

% time

Amount

Consultant fees, 25 days à US \$150.-
(according to the policy of the CTR)

US \$ 3.750.-

Social security

\$ 970.-

Technical

Administrative assistance

\$ 3.000.-

Social security

\$ 780.-

Sub-Total for A US \$ 8.500.-

B. Consumable supplies (by major categories)

None

Sub-Total for B

C. Other expenses (itemize)

Travel (6 round-trip tickets Zurich-
Stockholm, first class)

\$ 2.250.-

Per diem 25 days à US \$ 50.-

\$ 1.250.-

Phone, unforeseen costs, etc.

\$ 1.050.-

Sub-Total for C \$ 4.550.-

Running Total of A + B + C US \$13.050.-

D. Permanent equipment (itemize)

Sub-Total for D

E. Indirect costs (15% of A+B+C)

£

\$ 1.950.-

15. Estimated future requirements:

Total request

US \$15.000.-

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2						
Year 3						

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16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Lars Friberg, M.D.
 Signature *L. Friberg* Date Jan. 30, 1974
 Telephone 08/34 05 60 1165
Area Code Number Extension

Checks payable to

The Karolinska Institute

Responsible officer of institution

Typed Name Margareta Almling
 Title Chief Business Officer
 Signature *Margareta Almling* Date Jan. 30, 1974
 Telephone 08/34 05 60 1974
Area Code Number Extension

Mailing address for checks

Department of Environmental Hygiene
S-104 01 Stockholm 60, Sweden

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Department of Environmental Hygiene
S-104 01 Stockholm 60, Sweden

January 30, 1974

Appendix

Organization and coordination of collaborative twin research

This application concerns a research programme that is intended eventually to lead to a collaborative analysis of the results from twin studies performed in different countries. The programme may cover a period of two to three years, but the present application concerns only the first year. As a result of an effort, initiated by the Department of Environmental Hygiene of the Karolinska Institute and the Council for Tobacco Research - USA, Inc., to interest several countries to perform twin studies, representatives of research organizations from Australia, Belgium, Finland, Japan, New Zealand, Sweden, and the United States, agreed at a meeting in Miami, 1973, on some general principles in regard to a series of collaborative studies. A protocol from the meeting has already been submitted to the Council for Tobacco Research - USA, Inc.

According to the agreed organization plan the registry should be managed by a coordinating committee based initially on the Karolinska Institute, Department of Environmental Hygiene, and chaired by Professor Lars Friberg. The coordinating committee should, besides the Chairman, consist of the heads of each group contributing to the registry, as well as a number of independent experts, who are not contributors to the registry and who will rotate periodically. The activity of the coordinating committee should be to arrange meetings, consultations, coordinating of activities of participating groups, establishment of the registry itself, and analyzing the findings and preparing publications.

It is not possible to foresee today exactly to what extent the coordinating committee will be engaged during the first year. It can be expected, however, that besides the review of applications, some consultations will be needed in regard to practical problems in the different countries. It seems probable that the activity within the coordinating committee will be more demanding at the final stage, when the results are going to

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be analyzed. We do not expect that the whole committee, including the different contributors to the registry, will need to meet during the first year. For that reason we suggest that an executive committee be constituted immediately and consisting only of Professor Lars Friberg, Chairman, Dr. Rune Cederlöf, Executive Secretary, Professor Frederick H. Epstein, Consultant, and Dr. Zdenek Hrubec, Consultant. If the executives committee feels it necessary to seek further advice it should do so by correspondence with the other contributing researchers and/or with an appropriate consultant outside the group.

The financial resources needed for the first year can only be roughly estimated.

As for the activities of the executive committee, we are requesting a total of US \$15.000.-. One item is consultant fees to the members of the executive committee, or other consultants. We estimate that 25 men-days will be needed for reviewing the applications, discussing the research policy, giving directions in order to coordinate methods, etc. The consultant fees should only be used when special full-day meetings are set up between the members of the group, and according to the policy of the Council for Tobacco Research - USA, Inc. the fee has been fixed at \$150.-/day. We foresee that administrative assistance will be needed mainly for correspondence with the different registries. We expect that the different registries will, to a fairly high degree, ask for advice in many practical matters. We would like to be able to use the sum of \$15.000.- not only for secretarial help, but also for other consultations that may arise, e.g. in regard to coding principles, help with programming, etc. We have estimated the costs for travel at \$2.250.-. This sum is based on 6 round-trip tickets Zurich-Stockholm, first class, as one of the suggested members of the executive committee, Frederick H. Epstein, is living in Zurich. However, we would like to be able to use this sum for travel also for other European travel, if this is appropriate for the programme.

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We think it is important to emphasize that the nature of this project, to coordinate the activities of other research bodies, makes it difficult to foresee what other kinds of actions may be needed. First of all we would like to have the option to re-

distribute the total sum of \$15.000.- between the items A and C if this is of advantage to the programme. Secondly, we like to mention another possible cost that may arise during the year. In our preliminary discussions with the Council in December 1973, after the Miami meeting, we anticipated that some of the operating registries might wish to have a personal visit by Dr. Cederlöf in order to get some help with theoretical or practical matters. We suggested a sum of about \$10.000.- to cover European and/or overseas travel and consultant fees for such activities. We also suggested, at that time, that we ourselves should take the initiative to visit some of the countries in order to check up the proceedings. We think, today, that such an initiative may not be taken during the first year. If, however, some of the cooperating registries really demand to get help of this kind, and if the executive committee thinks that such help will be necessary for the success of the programme, Dr. Cederlöf will certainly comply with such a request. We think, however, that an activity of this kind should be discussed with the Council at each occasion and appropriately funded, if the Council agrees. We suggest, therefore, that the Council sets aside a sum of \$10.000.- for such activities that can be used at the discretion of the Scientific Director of the Council without the necessity in each case to seek further advice from the Board of Advisors.

1003545349

#971 - HONEYMAN

1003545350

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

February 13, 1974

Grant application No. 971

EPIDEMIOLOGY

To: The committee comprising Drs. Bing, Gardner, Lynch

Subject: Merton S. Honeyman, Ph.D., Connecticut State Department
of Health, Hartford
New application No. 971
"Environmental Influences on Like Sexed Twins"

History

CTR sponsored planning meeting on International Collaborative Twin Studies at Miami, December 10 - 14, 1973. Seven of the participants (from New Zealand, United States, Belgium, Sweden, - Australia, Japan and Finland) were invited to submit proposals. To date five have been received.

Request

For the first year \$54,940 is requested, plus two additional years at approximately \$80,000 each.

Document Submitted

Enclosed is application dated 1/29/74.

Comment

Dr. Carl Seltzer has been asked to evaluate these proposals.

JW
F.W.N.

FWN:wg
Encl.

1003545351

THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

FEB 1 1974

Date: 1/29/74

1. Principal Investigator (give title and degrees):

Merton S. Honeyman, B.A., M.S., Ph.D.

Director of Research, Office of Mental Retardation

Barbara Christine, B.S., M.D., Chief, Chronic Disease Control Section

2. Institution & address:

Connecticut State Department of Health

79 Elm Street

Hartford, Connecticut 06115

3. Department(s) where research will be done or collaboration provided:

Chronic Disease Control Section

Office of Public Health

Connecticut State Department of Health

4. Short title of study:

Environmental influences on like sexed twins

5. Proposed starting date: July 1, 1974

6. Estimated time to complete: 3 years

7. Brief description of specific research aims:

The specific aims of this project are to determine the morbidity and mortality experience of twins (25-59 years of age) in relation to their environmental exposure history. Smoking, alcohol consumption, and if possible air pollution exposure histories will be the environmental variables studied. To date two large scale twin studies (Karolinska Institute, Stockholm and the National Research Council) have investigated these variables but the results have not been significant due to small numbers in certain cells. It is the aim of this project to use a questionnaire very similar to that used by the two previous studies so that the data may be pooled in order to obtain sufficient numbers.

1003545352

8. Brief statement of working hypothesis:

2.

There is no difference in the incidence of morbidity of monozygotic and dizygotic twin pairs discordant for smoking history.

There is no difference in the incidence of morbidity between monozygotic and dizygotic co-twins, discordant for smoking history.

9. Details of experimental design and procedures (append extra pages as necessary)

The Connecticut Twin Registry contains a listing of all multiple births in Connecticut since July 1, 1897. Addresses of twins living in Connecticut are updated periodically, the last update was done in 1968.

In order to obtain the current addresses of a minimum of 2000 pairs of like-sexed twins, the 5440 pairs of like-sexed twins age 25-59 born in Connecticut who survived infant mortality will be searched for. This search involves use of city directories, obituary columns, etc. These resources have proved very successful in the past and it is estimated that at least 60% of the 5440 like-sexed pairs will be located with both living in Connecticut. If one is living in Connecticut, his or her twin's address can be obtained from this source.

Once the twins have been located a mail questionnaire (Appendix A) will be sent to each twin with a covering letter explaining the study as a part of an international collaborative effort. The questionnaire is a modification of the questionnaire used by the Karolinska Institute, Stockholm and the National Research Council (the Veterans Administration Twin Registry). For non-response to the first mailing, a second mailing will take place, and if this fails to obtain a response a telephone call to obtain cooperation will follow. The Karolinska Institute reports obtaining better than an 80% response rate utilizing this format.

The initial phase of updating addresses will take approximately one year to complete. Mailings can proceed as addresses are obtained. All mailings and telephone contacts should be completed by the end of the second year.

Twin pairs discordant for smoking histories and a sample of twin pairs concordant for smoking or non-smoking will be asked to have a physical examination. A sub-contract will be written with the Out-Patient Department of Hartford Hospital for these examinations. A complete history will be taken and a standard physical examination given including an electrocardiogram, blood studies, and appropriate x-rays.

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9. Details of experimental design and procedures (Cont.)

The data collected will then be analyzed for differences between co-twins and between monozygotic and dizygotic concordant and discordant pairs.

Long term follow-up on all twin pairs will be maintained and mortality data will be obtained from death certificates and hospital records.

Zygosity determination will be made by use of the questionnaire method using the question, "Were you as alike as two peas in a pod when you were growing up?" This question has been shown to be as good as blood group analysis on large populations, with accuracy approaching 95%.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Necessary space is available in the Chronic Disease Control Section for the purposes of this project.

11. Additional facilities required:

None

12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

1003545355

12.

Curriculum Vitae

Merton S. I

Address:

REDACTED

Marital Status:

Born:

REDACTED

REDACTED

Present Position and Employer: Director, Division of Research

Office of Mental Retardation

Connecticut State Department of Health

Education

B.A.	University of Connecticut, Storrs, Connecticut	Zoology
M.S.	University of Connecticut, Storrs, Connecticut	Zoology
Ph.D.	Ohio State University, Columbus, Ohio	Zoology (Genetics)

REDACTED
REDACTEDAdditional Training and Experience

Yale University School of Medicine	Postdoctoral Fellow	1954-57
Teachers College of Connecticut	Instructor - Evening College	1954-55
Hillyer College	Adjunct Assistant Professor	1955-56
National Cancer Institute	Geneticist	1957-59
Connecticut State Department of Health	Geneticist	1959-65
University of Hartford	Adjunct Assistant Professor	1960-
Yale University School of Medicine	Lecturer in Public Health - (Human Genetics)	1961-70
Connecticut State Department of Health	Director, Division of Program Analysis and Research, Office of Mental Retardation	1966-
Manchester Community College	Lecturer in Biology	1971-

Member of World Health Organization Expert Committee on Methodology of Twin Studies held at Geneva, Switzerland October 26 - November 1, 1965. Report published in Acta. Genet. Med. Gemell. 25:109, 1966.

Consultant to the Swedish Twin Registry, Karolinska Institute, Stockholm Sweden, May 1967.

Consultant to the Rheumatic Fever Clinic, Hadassah Medical Organization, Jerusalem, Israel on Family Study of Rheumatic Fever, November 1967.

Consultant to the Department of Medicine of Hartford Hospital and to Newington Children's Hospital.

Clinical Associate, Division of Medical Genetics, Department of Pediatric and Medical Specialties, University of Connecticut School of Medicine.

1003545356

Publications:

1. Crellin, E.S., and M.S. Honeyman. The Inhibition of Interpubic Ligament Formation by Progesterone In Pregnant Mice. *Ant. Rec.* 127:407, 1957.
2. Crellin, E.S., P.B. Wood, and M.S. Honeyman. Flexibility Changes of Pelvic Joints In Pregnant and Hormonally-Injected Mice. *Ant. Rec.* 127:408, 1957.
3. Crellin, E.S., and M.S. Honeyman. Pelvic Joint Changes during the Estrous Cycle in Mice. *Ant. Rec.* 127:408, 1957.
4. Honeyman, M.S., A Quantitative Genetic Study of a Nutritional Strain Difference in Mice. *J. Heredity* 48:84, 1957.
5. Greenberg, R.A., and M.S. Honeyman. Lung Cancer: A Growing Problem. *Connecticut Health Bulletin* 73:269, 1959.
6. Honeyman, M.S. The Connecticut Twin Registry. *Connecticut Health Bulletin.* 74:111, 1960.
7. Honeyman, M.S. The Recent Advances In Human Cytogenetics. *Connecticut Health Bulletin.* 75:83, 1961.
8. Honeyman, M.S. Genetics and Disease. *Connecticut Health Bulletin.* 75:191, 1961.
9. Honeyman, M.S. and E. Siker. Cystic Fibrosis of the Pancreas. *Connecticut Health Bulletin.* 75:275, 1961.
10. Ballar, J.C. III, M.S. Honeyman, and H. Eisenberg. Incidence and Mortality Rates for Leukemia and Lymphoma. *Public Health Reports.* 77:281, 1962.
11. Honeyman, M.S. The Familial Implications of Breast Cancer In Man. *Connecticut Health Bulletin.* 76:351, 1962.
12. Honeyman, M.S., and E. Siker. Cystic Fibrosis of the Pancreas. An Estimate of the Incidence Rate. *Am. J. Hum. Genet.* 17:461, 1965.
13. Wetstone, H.J., M.S. Honeyman, and R. B. McComb. Genetic Control of the Quantitative Activity of a Serum Enzyme In Man. *J.A.M.A.* 192:1007, 1965.
14. Eisenberg, H., M.S. Honeyman, H.S. Barrett. A Twin Family Study of Blood Pressure. Presented at the American Heart Association Annual Meeting, Atlantic City, New Jersey, October 23, 1964.
15. Honeyman, M.S., H. Eisenberg, and F.M. Foote, The Twin Study of Serum Cholesterol. Presented at the American Public Health Association Annual Meeting, New York, New York. October 6, 1964.

1003545357

16. Eisenberg, H., M.S. Honeyman, and R.B. Connolly. Trends In Female Breast Cancer. Connecticut, 1935-1959. Presented at the American Public Health Association Annual Meeting, New York, New York. October 6, 1964.
17. Foote, F.M., H. Eisenberg, and M.S. Honeyman. Trends In Cancer Incidence and Survival In Connecticut. Presented at the American Medical Association Annual Meeting, San Francisco, California. January 24, 1964.
18. Brewer, G.J., J.C. Gall, M.S. Honeyman, H. Gershowitz, R.J. Dern, C.G. Hames. Inheritance of quantitative expression of G-6-PD deficiency in heterozygous Negro females - A twin study. Clin. Res. 13:265 (abst) 1965.
19. Foote, F.M., H. Eisenberg, and M.S. Honeyman. Trends in Cancer Incidence and Survival in Connecticut. Cancer 19:1573, 1966.
20. Reznikoff, M., and M.S. Honeyman. MMPI Profiles of Monozygotic and Dizygotic Twin Pairs. J. Consult. Psych. 31:100, 1967.
21. Brewer, G.J., J.C. Gall, M.S. Honeyman, H. Gershowitz, R.J. Dern, and C.G. Hames. Inheritance of Quantitative Expression of the X-linked gene Glucose-6-phosphate Dehydrogenase Deficiency, in Heterozygous Negro Females - A Twin Study. Biochem. Genet. 1:41, 1967.
22. Shreffler, D.C., G.J. Brewer, J.C. Gall and M.S. Honeyman. Electrophoretic Variation in Human Serum Ceruloplasmin: A New Genetic Polymorphism. Biochem. Genet. 1:101-115, 1967.
23. Shreffler, D.C., G.J. Brewer, J.C. Gall, and M.S. Honeyman. Genetic Polymorphism in Human Serum Ceruloplasmin. pp. 91-92. 3rd Intern. Congr. Human Genet., Abstr. of Contrib. Papers. 1966.
24. Honeyman, M.S., H. Eisenberg, and J.T. Morrison. A Twin Study of Serum Cholesterol Level. pp. 47-48. 3rd Intern. Congr. Human Genet., Abstr. of Contrib. Papers. 1966.
25. Rappaport, H., M. Reznikoff, B.C. Glueck, M.S. Honeyman, and H. Eisenberg. Smoking Behavior in Offspring of Heart Disease Patients: A Response to Cognitive Dissonance. J. Consult. Psych. 32:494-496, 1968.
26. Honeyman, M.S., H. Rappaport, M. Reznikoff, B.C. Glueck and H. Eisenberg. Psychological Impact of Heart Disease in the Family of the Patient. Psychosomatics. 9:34-37, 1968.
27. Honeyman, M.S. and I. Gabrielson. Public Health Aspects of Genetic Screening. Birth Defects Original Article Series, Vol. IV, No. 6; Nov. 1968.
28. Finn, Fred F., Bert W. Schmickel, and Merton S. Honeyman. The Seaside Story. Connecticut Health Bulletin. 82.4 (April) 1968.
29. Honeyman, M.S. and B.W. Schmickel. Progress in Human Genetics As Related To Mental Retardation Services. Connecticut Health Bull. 83.2 (February) 1969.
30. Vanderkolen, R., G.J. Brewer, M.S. Honeyman, J. Morrison, and S.W. Hoobler. A Study of Hypertension in Twins. American Heart Journal. 79:454-457, 1970.

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31. Dies, R., M. Reznikoff, M. Honeyman and C. White, Personality and Smoking Patterns In a Twin Population. J. Proj. Tech. Person., 1969, 33, 457-463.
32. Honeyman, M.S. and E. Davis. A Genetic Study of Rheumatic Fever Clustering In Families. Acta. Genet. Med. Gemellol, 1971, 20:189-198.
33. Honeyman, M.S. Sickle Cell Anemia - An Inherited Disease. Connecticut Health Bulletin, 85, 3 (March) 1971.
34. Siker, E., M.S. Honeyman, V. Bapat, A.D. Crabtree, and V. Shannon. A Sickle Cell Disease Program--Planning, Education, Genetic Counseling. Connecticut Health Bulletin. 86:255-261, 1972.
35. Reznikoff, M., G. Domino, C. Bridges, and M.S. Honeyman. Creative Abilities In Identical and Fraternal Twins. Behavior Genetics. In Print 1973.
36. Reznikoff, M., G. Domino, C. Bridges and M.S. Honeyman. Perceptions of Alikehood and Attitudes toward Being A Twin: Comparison of Identical and Fraternal Twin Pairs. Perceptual and Motor Skills. 37:103-106, 1973

1003545359

CURRICULUM VITAE

Barbara W. Christens, M.D.

Date of Birth:

Place of Birth:

Soc. Sec. No.

Education

Saratoga College, Saratoga, New York - Bachelor of Arts degree

Albany Medical School, Albany, N.Y. - Doctor of Medicine degree

Watertown Hospital, Watertown, Conn. -

Fellow in New Haven Rheumatic Fever Clinic, Grace-New Haven Hospital,
New Haven, Conn. -Fellow in Cardio-Respiratory Laboratory, Hartford Hospital,
Hartford, Conn. -Yale School of Public Health, New Haven, Conn. -
Master in Public Health degree 1961

Employment

Assistant in General Practice to Dr. B. Walker, Torrington and
Cornwall, Connecticut - 1952-1952

Practice in General Medicine, Torrington, Connecticut - 1953-1960

Chief, epidemiology section, Conn. State Dept. of Health - Oct. 1961 to
March 25, 1963

Director of Health, Groton, Conn. - May 1963 to July 1968.

Chief, epidemiology section, Conn. State Dept. of Health - August 9, 1968
to October 21, 1968Chief, chronic disease control section - Conn. State Dept. of Health -
November 1968 to present

3/11/73

1003545360

Barbara W. Christine

Publications

1. _____, and staff of the Chronic Disease Control Section: Cancer in Connecticut 1965, Monograph published 1969.
2. _____, Sullivan, P., and Connelly, R.: Cancer in Connecticut 1966. Connecticut Health Bul., 84:5, May 1970.
3. _____, Sullivan, P., Connelly, R., Foote, F.: Cancer incidence, mortality and survival in Connecticut during the 1960's. Connecticut Medicine, 34:8, 564-569, Aug. 1970.
4. _____, Sullivan, P.D.: Effects of cigarette smoking on Connecticut residents. Connecticut Health Bul. 85:1, Jan. 1971.
5. _____, Flannery, J., Connelly, R.: Cancer in Connecticut 1967. Conn. Health Bul. 85:1, Feb. 1971.
6. _____, Flannery, J., Sullivan, P.: Cancer in Connecticut 1968. Conn. Health Bul. 85:11, Nov. 1971.
7. Shanmugaratnam, K., Muir, C.S., Tow, S.H., Cheng, W.C., Christine, B., and Pedersen, E.: Rates per 100,000 births and incidence of chorioepithelioma and malignant mole in Singapore Chinese and Malays - comparison with Conn., Norway and Sweden. Inter. J. of Cancer:8, 165-175 (1971).
8. Schoenberg, B.S., Christine, B.: Neoplasms of the brain and cranial meninges - A study of incidence, epidemiological trends, and survival. Presented at the 22nd annual meeting of the Am. Acad. of Neurology, April 30-May 2, 1970.
9. Connelly, R.P., Christine, B.: Cancer following infectious mononucleosis. Presented at the 10th International Cancer Congress in May 1970.
10. Groff, W.H., Pitt, T.H., Christine, B.W.: Cervical cancer and social rank in metropolitan Connecticut. Agricultural Experiment Station Bulletin. Research Report 34, May 1971.
11. Cutler, S.J., Christine, B., Barclay, T.H.C.: Increasing incidence and decreasing mortality rates for breast cancer. Cancer 28:6, Dec. 1971.
12. Sullivan, P.D., Christine, B.W., Connelly, R.R., and Barrett, H.: Analysis of trends in age adjusted incidence rates of cancer for ten major sites, 1935-1965. The Am. J. of Pub. Health 62:8, 1065-1071, August 1971.
13. _____, Flannery, J.T., Sullivan, P.D.: Cancer in Connecticut 1969. Conn. Health Bul. 86:4, April 1972.
14. _____, Sullivan, P.D., Flannery, J.T.: Cancer in Connecticut 1970. Conn. Health Bul. 86:11, Nov. 1972.
15. Kryscio, R.J., Myers, M.H., Prasiner, E.T., Hoise, H.W., Christine, B.W.: A study of the space-time distribution of Hodgkin's disease in Connecticut, 1940-69. To be published.

1003545361

Barbara W. Christine - Publications

16. O'Connor, G.T., Correa, T., Christine, B., Axtell, L., Myers, M.: Hodgkin's disease in Connecticut: Histology and age distribution. To be published in J. of Nat. Cancer Inst.
17. Groff, W., Pitt, T., and Chapelle, M.: The relationship of incidence of cervical cancer and socioeconomic status in seven cities, 1959-1964. Connecticut Medicine, 35:2, Feb. 1972.
18. Chapelle, M., Nadomi, D.: Cancer of the cervix in Connecticut. Connecticut Medicine 35:12, 669-680, Dec. 1972.
19. Andrews, D., Christine, B.: Leukemia surveillance in Connecticut 1970. To be published in Conn. Health Bul. 87:4, April 1973.
20. Kuehn, P.G., Christine, B., and Coley, G.M.: Carcinoid tumors - A study of 550 cases. To be presented at the 1973 spring meeting of the James Ewing Society and published in Cancer.
21. Curnen, M.G., McCrea, Vanna, A., Christine, B., and Turgeon, L.: Childhood leukemia and maternal influenza: Are they related? To be presented at the meeting of the Society of Epidemiologic Research in Winnipeg, Canada, June 21-23, 1973.
22. Odell, R., Sullivan, P., Christine, B.: Mortality from diseases of the respiratory system - Conn. 1960-1971. Conn. Health Bul. 87:2, Feb. 1973.
23. Sullivan, P., Flannery, J.: Cancer in Connecticut 1971, Conn. Health Bul. 87:8, August 1973.
24. Schoenberg, B.S., Fraumeni, J.F. Jr., Greenberg, R.A., Christine, B.: Multiple primary malignancies in Connecticut 1935-1964: Clues to etiology. To be published in the Proceedings of the Perugia Conference on Multiple Primary Malignant Tumors. Submitted 5-73.
25. Sullivan, P., Flannery, J.: Cancer in young Connecticut adults, aged 15-29 years, 1950-1969. To be published in the proceedings of the Second International Symposium on Cancer Detection and Prevention.
26. Axtell, L., Myers, M., Christine, B., and Linden, G.: Trends in survival and classification for leukemia patients diagnosed 1940-69. To be submitted to Blood.
27. Flannery, J., Christine, B., Foote, F.: Problems of maintaining and updating a tumor registry. Presented at the International Association of Cancer Registry Symposium in Dusseldorf, Germany, April 6, 1973.
28. Honeyman, M., Christine, B., Wetstone, S.: Investigation of cancer in mothers of retarded individuals.
29. Cramer, D.W., Cutler, S.J., Christine, B.: Trends in the incidence of endometrial cancer in the United States.

3/24/73

1003545362

13. Publications:

Merton S. Honeyman, Ph.D.

Wetstone, H.J., M.S. Honeyman, and R.B. McComb. Genetic Control of the Quantitative Activity of a Serum Enzyme in Man. J.A.M.A. 192:1007, 1965.

Rappaport, H., M. Reznikoff, B.C. Glueck, M.S. Honeyman, and H. Eisenberg. Smoking Behavior in Offspring of Heart Disease Patients: A Response to Cognitive Dissonance. J. Consult. Psych. 32:494-496, 1968.

Honeyman, M.S., H. Rappaport, M. Reznikoff, B.C. Glueck and H. Eisenberg. Psychological Impact of Heart Disease in the Family of the Patient. Psychosomatics. 9:34-37, 1968.

Dies, R., M. Reznikoff, M. Honeyman and C. White. Personality and Smoking Patterns in a Twin Population. J. Proj. Tech. Person., 1969, 33, 457-463.

Reznikoff, M., G. Domino, C. Bridges, and M.S. Honeyman. Creative Abilities in Identical and Fraternal Twins. Behavior Genetics, Vol. 3, No.4, 1973.

Barbara Christine, M.D.

Christine, B., Flannery, J.T., Sullivan, P.D.: Cancer in Connecticut 1969. Conn. Health Bul. 86:4, April 1972.

Christine, B., Sullivan, P.D., Flannery, J.T.: Cancer in Connecticut 1970. Conn. Health Bul. 86:11, Nov. 1972.

Christine, B., Groff, W., Pitt, T., and Chapple, M.: The relationship of incidence of cervical cancer and socioeconomic status in seven cities, 1959-1964. Connecticut Medicine, 36:2, Feb. 1972.

Christine, B., Chapple, M., Nadeau, D.: Cancer of the cervix in Connecticut. Connecticut Medicine 36:12, 669-680, Dec. 1972.

Christine, B., Sullivan, P., Flannery, J.: Cancer in Connecticut 1971, Conn. Health Bul. 87:8, August 1973.

1003545363

14. First year budget:

Salaries (give names or state "to be recruited")	% time	Amount				
Professional (give % time of investigator(s) even if % salary requested)						
Merton S. Honeyman, Ph.D.	10	REDACTED				
Barbara Christine, M.D.	5	REDACTED				
Geneticist (Supervisor)	100	REDACTED				
"to be recruited"						
Technical						
2 Clerk II	100	REDACTED				
1 Typist II	100	REDACTED				
Fringe Benefits 30.15%		REDACTED				
Sub-Total for A		REDACTED				
B. Consumable supplies (by major categories)						
Printing		2,000				
Office Supplies		500				
Sub-Total for B		2,500				
C. Other expenses (itemize)						
Postage		2,000				
Telephone		500				
Travel		500				
Sub-Total for C		3,000				
Running Total of A + B + C		47,244				
D. Permanent equipment (itemize)						
1 Electric Typewriter		400				
1 5 Drawer File Cabinet		85				
1 Electronic Calculator		125				
Sub-Total for D		610				
E. Indirect costs (15% of A+B+C)		7,086				
Total request		54,940				
15. Estimated future requirements:						
	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	B	0	25,500	-	10,475	80,306
Year 3	B	500	25,500	-	10,803	82,825

1003545364

List financial support from all sources, including own institution, for this and related research projects.

Source
(grant numbers)

PENDING OR PLANNED

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Typed Name Merton S. Honeyman, Ph.D.

Signature M. T. G. L. Date 1/20/74

Telephone _____ **REDACTED** _____
 / Area Code Number Extension

Connecticut State Department of Health

Typed Name Harold S. Barrett, M.D.

Title Deputy Commissioner

Signature Harold E. Bennett Date 1/20/74

Telephone REDACTED REDACTED
 Area Code Number Extension

Mailing address for checks

Business Office - 79 Elm Street

Hartford, Connecticut 06115

1003545365

#965 KJELLSTROM

1003545366

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

- February 13, 1974

Grant application No. 965

EPIDEMIOLOGY

To: The committee comprising Drs. Gardner, Jacobson, Lynch

Subject: Tord Kjellstrom, M. E., M.B., Karolinska Inst., Sweden
New application No. 965
"Pilot Study for Setting up a Registry of Twins Reared
Apart Since Birth"

History

CTR sponsored planning meeting on International Collaborative Twin Studies at Miami, December 10 - 14, 1973. Seven of the participants (from New Zealand, United States, Belgium, Sweden, Australia, Japan and Finland) were invited to submit proposals. To date five have been received.

Request

For the first year No. 965 requests \$12,756, plus one additional year.

Document Submitted

Enclosed is application dated 1974-01-24, with appendices A, B, and C.

Comment

Dr. Carl Seltzer has been asked to evaluate these proposals.

JWM
F.W.N.

FWN:wg
Encl.

1003545367

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8385

Application for Research Grant
(Use extra pages as needed)

Date: 1974-01-24

1. Principal Investigator (give title and degrees): Tord Kjellström, M.Eng., M.B.

2. Institution & address: Department of Environmental Hygiene
Karolinska Institute
104 01 Stockholm 60

3. Department(s) where research will be done or collaboration provided:

Toyama Prefecture Institute of Hygiene and Medical Microbiology
1-15, Ohtemachi, Toyama City, Toyama Prefecture
Japan
Director: Dr Kentaro Kubota

4. Short title of study:

Pilot study for setting up a registry of twins reared apart since birth.

5. Proposed starting date: April 1, 1974

6. Estimated time to complete: 1 year

7. Brief description of specific research aims:

Epidemiological studies comparing dizygotic and monozygotic twins discordant within the pairs for some environmental agent (e.g. smoking habits) are powerful for assessing effects or dose-response relationships as the influence of genetic factors can be held under control. However, it has been argued that even in such studies there is still no clearcut division between environment and genetics because twins usually have had very similar environment during childhood when they live together. As twins reared apart from birth are usually very scarce even a small number of such twin pairs could be a useful population for studying concordance rates in behavioural factors (smoking, drinking, stress, personality, type of work, etc.) and clinical indicators (blood pressure, cholesterol in blood, alpha-anti-trypsin, etc.) with pertinence in relation to chronic diseases.

An unique feature of a twin registry in Japan is the possibility of finding twins reared apart from birth. In the past it was not uncommon that twins were separated at birth by one of them being placed in another family.

There may be problems in identifying such twin pairs as efforts were taken to keep the fact of the twin birth secret to the twins themselves. The aim of this pilot study is to investigate how those problems can practically be solved and also, if possible, to set up a registry of twins from Toyama Prefecture and adjacent areas. The goal for this pilot

cont...

7. cont..

registry is 100 complete, living, monozygotic twin pairs reared apart. Other complete twin pairs found as a part of the compilation procedure will be included. If the procedures adopted do not produce any twins reared apart after an initial test period, the project will be stopped and most of the grant will be reimbursed to the Council.

The project will be designed such as to fit into the "International Collaborative Twin Registry".

1003545369

8. Brief statement of working hypothesis:

Japanese twins reared apart from birth may be identified through existing administrative records and their present address may be localized.
A limited twin registry consisting of such twins may be set up in Toyama.

9. Details of experimental design and procedures (append extra pages as necessary)

Every family head in Japan has since 1870 a family record, Koseki, where all vital data about the family is included. When a man marries he gets his own Koseki. Births were reported by the family head to the town office and thus included in the Koseki. Every move of an individual registered in the Koseki to another family's Koseki is included.

Since 1945 all births are reported in birth records, Shusseitodoke. From the Shusseitodoke a person can be linked to a Koseki and thus a separation of twins after birth may be identified.

From the Koseki linkage can be accomplished to a registry of that person's present address.

The problem of setting up the twin registry will be attacked from four different directions:

1. Contact will be taken with Japanese scientists making psychosocial studies on twins in order to find twin pairs reared apart from birth, that might be included in this registry.
2. In an epidemiological study on 5,000 women from Toyama in the age groups 45-65 a question about twins in the family is asked by an interviewer. In February, 1974, the answers will be compiled. It can be estimated that an average of 200-300 twin births should exist in this material. Many of them would be the same as those found in procedures 3 and 4 and thus the work of going through family and birth records may be diminished.
3. The family records for men born in Toyama 1880-1900 and 1915-1925 will be gone through until a total of 400 twin births in the first group has been found and 200 in the second group. For each family record the estimated average number of birth is five and the number of twin births per 1,000 births is estimated to six. This means an expected 30 twin births per 1,000 family records. 20,000 such records would have to be gone through to reach the goal above.

The birth records for twins with parents in the 1915-1925 age group will be gone through as a cross-check (twins born after 1945). Apart from this 5,000 randomly selected birth records covering the years 1945-1950 from the same area as the selected family records will be gone through (estimated number of twin birth = 30) in order to get an idea of the prevalence of twin births not being included in the family records.

cont...

1003545370

9. cont..

The estimated time necessary for looking through one record is two minutes. To go through 25,000 records would take 50,000 minutes, which for two persons means about 400 hours or 12 working weeks when travel time is included.

The procedure above is aimed at finding about 600 twin births in two generations. Approximately half of them will be monozygotic. By linking the data together in this way it will become clear which twin pairs have been reared apart from birth.

The procedures 1 and 2 can be performed at low costs and will be pursued completely. The other procedures will be started on a small scale in order to see if there is reason to believe that twins reared apart can be found at all, and if the goal for this pilot study can be achieved. If the result of this "pilot - pilot" study is negative the project will be stopped and the expenditures will be maximum US\$2,000.

If the result is positive the next step is to find out if the twins found are still alive and to find their present address. The family record of the twins themselves will have to be located and to find addresses the registries used for general elections may be used. This work will for the 600 twin pairs cover approximately another 12 working weeks for two persons. In this pilot study the zygosity of twins found will not be studied and neither will any interviews or clinical tests be done.

If this method for setting up a registry of twins reared apart is successful, in the second year studies of zygosity in combination with questionnaire (by interview) and clinical examination would be performed at a cost of about US\$50 per twin. If 100 monozygotic and 100 dizygotic same-sex pairs are examined the cost would be US\$20,000 + administrative costs. If the resources would be more limited, only zygosity and questionnaire studies would be done at a cost of about US\$20 per twin. The total cost would then be US\$8,000.

1003545371

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Most of the work is clerical and will be done in the offices of the towns selected in Toyama.

For the administration of the project the facilities of the Department of Environmental Hygiene, Karolinska Institute and the Institute of Hygiene and Medical Microbiology in Toyama will be available.

11. Additional facilities required: None

1003545372

12. Biographical sketches of investigator(s) and other professional personnel (append): Appendix A + B

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available). Appendix C

14. First year budget:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s)
even if no salary requested)

% time

Amount

Supervisors: Tord Kjellström,
Kentaro Kubota)

5

Research assistant, Sweden
(Ulla Lorch, B.A.)

5

Research assistant, Japan
(to be recruited)

5

Technical

Two clerical assistants, Japan
(to be recruited)

50

REDACTED
REDACTED

REDACTED

Sub-Total for A

REDACTED

B. Consumable supplies (by major categories)

Sub-Total for B

0

C. Other expenses (itemize)

Travel costs Sweden-Japan

4,000

Travel within the investigation area

500

Computer programming and card punching

500

Administrative costs within Japan

1,000

Sub-Total for C

6,000

Running Total of A + B + C

11,000

D. Permanent equipment (itemize)

Sub-Total for D

0

E. Indirect costs (15% of A+B+C)

E

1,650

Total request

12,650

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	B		25,000 (8,000)		4,000	31,000
Year 3						(11,500)

1003545373

16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project

Source
(give grant numbers)

Amount

Inclusive
Dates

PENDING OR PLANNED

Title of Project

Source
(give grant numbers)

Amount

Inclusive
Dates

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Tord KjellströmSignature Tord Kjellström Date 1974-01-23

Telephone

REDACTED

Area Code

Number

Extension

Responsible officer of institution

Typed Name Margareta AmlingTitle Head of AdministrationSignature Margareta Amling Date 1974-01-23

Telephone

REDACTED

Area Code

Number

Extension

Checks payable to

☒ Department of Environmental Hygiene

Mailing address for checks

Karolinska Institute

Pöck 104 01 Stockholm 60 Sweden

1003545374

Appendix A

CURRICULUM VITAE

Tord Kjellström

Born: **REDACTED**

Present employment: research assistant, Department of Environmental Hygiene, Karolinska Institute, Stockholm, Sweden, since 1971.

Mailing address: Department of Environmental Hygiene,
Karolinska Institute
104 01 Stockholm 60"
Sweden
telephone No 08/23 69 00

Education: graduated from Norra Latin High School,

REDACTED

Degree of Bachelor of Medicine from the Karolinska Institute, Stockholm,

REDACTED

Degree of Master of Mechanical Engineering from the Royal Institute of Technology, Stockholm,

REDACTED

Research student at the Department of Mathematical Engineering, Faculty of Engineering, Tokyo University, during 1968.

Passed requirements for Ba in the Japanese language at the Department of Japanese, Stockholm University, March 1969.

Earlier positions: research worker at the National Clinic of Assessment of Work Capacity, during 1966.

Research assistant at the Department of Social Medicine, Faculty of Medicine, Uppsala University, during 1969 and 1970.

1003545375

CURRICULUM VITAE

Ulla Lorch

Born: **REDACTED**

Present employment: Research assistant, Department of Environmental Hygiene, Karolinska Institute, Stockholm, Sweden, since May, 1969.

Mailing address: Department of Environmental Hygiene
Karolinska Institute
104 01 Stockholm 60
Sweden
telephone No 08/23 69 00

Education: Graduated from Adolf Fredrik High School,

REDACTED

Degree of Bachelor of Arts from the Stockholm University,

REDACTED

Subjects: major, sociology
minors, statistics and
political science

Publication: Friberg, L., Cederlöf, R., Lorch, U., Lundman, T., DeFaire, U.: Mortality in Twins in Relation to Smoking Habits and Alcohol Problems.

Arch. Environ. Health, 27, 294-304, 1973.

1003545376

LIST OF PUBLICATIONS OF TORD KJELLSTRÖM.

1. Kjellström, T.: A Mathematical Model for the Accumulation of Cadmium in Human Kidney Cortex. Nord. Hyg. Tidskr., p 111-119, 1971.
2. Smedby, B., Kjellström, T., and Berfénstam, R.: The Users of Hospital Care (Sjukhusvårdens Konsumenter) Sjukvårdens och Socialvårdens Planerings- och Rationaliseringsinstitut, report No 18, Stockholm, 1972 (in Swedish with summary in English).
3. Kjellström, T., and Friberg, L.: Interpretation of Empirically Documented Body Burdens by Age of Metals with Long Biological Half-Lives with special reference to past changes in exposure. Proceedings, 17th International Congress on Occupational Health, Buenos Aires, September 1972 (in press).
4. Kjellström, T.: An Epidemiological Study on Exposure and Effects of Cadmium. The general and industrial environment of a Swedish lead and copper smeltery. (Epidemiologisk expositions- och effektstudie av kadmium. En undersökning av den allmänna och industriella miljön vid Rönnskärsverken). Department of Environmental Hygiene, National Environmental Protection Board, August 1973 (in Swedish).
5. Friberg, L.F., Piscator, M., Nordberg, G.F., and Kjellström, T.: Cadmium in the Environment, 2nd edition, Chemical Rubber Company Press, Cleveland. Ohio (in press).

1003545377

#941 - LYNCH

1003545378

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

October 19, 1973

Grant application No. 941

EPIDEMIOLOGY

To: The committee comprising Drs. Gardner, Huebner, and Jacobson

Subject: Henry T. Lynch, M.D., Creighton University School of Medicine,
Omaha
New application No. 941
"Hereditary Factors, Cigarette Smoking, and Cancer Incidence
in 3,261 Families."

History

This proposal initially came to CTR as Case No. 152; application was encouraged.

Dr. Lynch has served CTR as a consultant and site visitor in evaluating applications. He has also visited our office and conferred with scientific staff.

Request

Application No. 941 requests \$30,360 plus one additional year.

Documents Submitted (attached)

1. Application (undated) received by CTR September 6, 1973, with Appendix, Tables and Figures.
2. Condensed biographical sketch of Henry T. Lynch (his full C.V. is available on request; but be warned it is voluminous).
3. Reprints of papers with Dr. Lynch as senior author, identified as numbers 9, 11 and 12 in his biographical sketch. Also a reprint of "Tumor Variation in Families . . ." by Lynch, et al, JAMA 222 1631, 1972.


F.W.N.

FWN:wg
Encls.

1003545379

THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8985

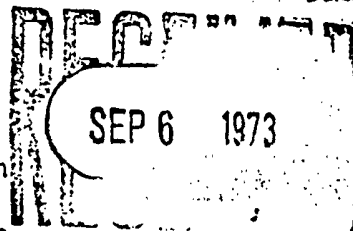
#941

Part I

Application for Research Grant

Date:

(Use extra pages as needed)



1. Principal Investigator (give title and degrees):

Henry T. Lynch, M.D., Professor and Chairman
Department of Preventive Medicine & Public Health
Hoda Guirgis, Ph.D., Assistant Professor
Department of Preventive Medicine & Public Health

2. Institution & address:

Creighton University School of Medicine
2500 California Street
Omaha, Nebraska 68178

3. Department(s) where research will be done or collaboration provided:

Department of Preventive Medicine and Public Health

4. Short title of study:

Hereditary Factors, Cigarette Smoking, and Cancer Incidence in 3,261 Families.

5. Proposed starting date: 9/1/73

6. Estimated time to complete: 2 years

7. Brief description of specific research aims:

- a). To evaluate smoking histories in a population characterized by familial proneness to cancer or familial absence of cancer. This population represents the first example of ascertainment of family histories of cancer from a large number of normal probands.
- b). To determine the status of heredity and its interaction with cigarette smoking history with objective evidence of cancer occurrences (all sites) in the several categories of cancer proneness versus cancer resistance, i.e., families with complete absence of cancer, families with one first degree relative with cancer, and families with two or more first degree relative with cancer.
- c). Statistical analysis will be made in search for correlations with specific histologic varieties of cancer and to relate this issue to the family history of cancer in the presence or absence of cigarette smoking.
- d). Diet, alcohol consumption, occupational exposures, and any cultural idiosyncrasies will be evaluated in context with their potential etiologic influences as contributory factors in carcinogenesis.

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8. Brief statement of working hypothesis:

We propose that an individual's genetic endowment is an important determinant in susceptibility to specific carcinogens. Since liability or proneness to a disorder is a function of interacting environmental and genetic factors, one would expect to find a variation in cancer-resistant and cancer-prone families with a change in either factor. If two families come from environments which are not strikingly different from each other, but show a variation in proneness to cancer one can assume that the major differentiating variable is the genetic factor. Thus, an individual's predisposition or resistance to a particular disease process depends upon that individual's genetic norm or range of reaction and the environmental factors interacting with his genotype.

Should this hypothesis prove to be correct, it would follow that cigarette smoking may pose an unusual health hazard to certain patients in a manner not unlike the current hypothesis suggesting an acceleration of the clinical course of emphysema in individuals who are cigarette smokers and who harbor genes for alpha₁ antitrypsin deficiency. Therefore, this hypothesis may in part explain the fact that the overwhelming majority of smokers do not contract lung cancer (or any other cancers). On the other hand, those cigarette smokers who do develop carcinoma of the lung and/or other cancers may represent a genetically susceptible population. Some of these patients would undoubtedly develop cancer spontaneously in the absence of cigarette smoking though they may have been subjected to other environmental carcinogens.

9. Details of experimental design and procedures (append extra pages as necessary)

Our population is unique in that it represents the first major effort in defining the frequency of cancer of all anatomic sites in a large number of families ascertained through evaluation of apparently healthy individuals. The initial studies were performed independently at two geographically distinct medical centers and were based upon 4,283 subjects from which over 5,000 families were generated. The statistical analysis showed virtually identical overall findings from these two populations (Tables 1-4 and Figures 1-4). The study group from which our samples will be derived will comprise 3,261 Nebraska families of which our investigators have established personal contact through our cancer screening activities of the probands. Thus, rapport has been well established, contributed to in part by the fact that a service, namely cancer screening was provided to all of these individuals. Finally, the activities of the research team with this group are current in that the screening activities have been conducted within the last two years.

The number of probands screened at the mobile cancer detection unit in eight locations is listed in Table 5. The geographic distribution of these locations in the state of Nebraska are shown in Figure 5. As described in Table 1, we find that 46.6 percent of the probands show no cancer history in their first degree relatives. We also find 29.3 percent had a first degree relative affected with cancer and 26.1 percent had 2 or more first degree relatives with a history of cancer.

In this study we will collect data on probands and their first degree relatives only, i.e., parents of probands, siblings of probands, and children of probands.

Random sampling and groups under study:

The method described by Goldstein (1964) will be adopted in this study. That is, each proband will be assigned a number; using the random tables we then begin filling the groups according to the sequences of numbers in the table.

Using this method the following groups will be selected:

(Group A) 200 probands having no history of cancer in first degree relatives selected from the 46.6% of total probands.

(Group B) 100 probands will be drawn from the pool of probands that had a history of one first degree relative affected with cancer selected from the 29.3% of all probands.

(Group C) 100 probands will be drawn from these probands that had 2 or more first degree relatives that had cancer selected from the 26.1%. The total number of probands will then be 400.

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Letters will be sent to each of the 400 probands explaining the nature of this study, and asking for their participation in it. A questionnaire will be included so that prior to an interview each proband will complete the following information:

1. Names and addresses of all first degree relatives.
2. Medical history authorization forms.
3. Additional medical history since the date of initial screening at the mobile unit.
4. Smoking and family history questionnaire to be completed by each first degree relatives following interviews with probands.

In each interview, details of smoking history including the specific type of smoking (cigarettes, cigars, pipe, chewing tobacco) including onset of smoking, duration, continuous versus discontinuous pattern, and so on, will be obtained on the specific patient being interviewed as well as the knowledge of smoking habit patterns in his or her first degree relatives. Thus, through interviewing a minimum of three patients from each family (the patient seen in the clinic and two or more first degree relatives), in addition to material from questionnaires in all first degree relatives we would have available to us sufficient information to cross check and thereby validate our historical information.

The effects of diet or tumor formation particularly in the gastrointestinal tract has resulted in intensive etiologic speculation in the world literature in recent years, since variations in tumor incidence among different nationality groups could be explained by dietary and perhaps other as yet unknown environmental effects. As a result of increasing affluence, the American diet is noted both for its high meat content and the use of convenience foods--most of which are of low bulk and highly refined. Of increasing concern has been the use of herbicides and pesticides in efforts to increase crop production; these may have possible effects on humans. Although an extensive dietary history might contain too many variables and may be too difficult to elicit, major differences in food intake may become evident by determining the patient's individual preference from food lists, as well as a rough estimate of the amount consumed. For all practical purposes, we propose to simplify our dietary history by dividing foods into four groups: 1) a meat-poultry-egg group; 2) cheese and dairy products; 3) bread and cereals; 4) fruit and vegetables. In the first part of the history, an effort will be made to determine the patient's food preferences; in the second part, an attempt will be made to evaluate size of servings, and the number of meals consumed per day. Since studies on food consumption have been published by the Department of Agriculture yearly, extending back to 1848, an attempt will be made to compare our patient's food intake with those compiled by the Department of Agriculture, and to investigate any major variation which might be found.

Following completion of the questionnaire and interview material on each family, the data will be reviewed by our Genetic Study Group Research Team, it will be analyzed, and a pedigree will be constructed. As with previous studies of this nature by our group, the amount of data obtained will require an extensive filing system, with appropriate clerical assistance. The use of a computer is also projected for the statistical evaluation of the data. These facilities are available to us.

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There are several possible results which might be achieved from this study as follows:

1. There may be no difference in the smoking history in the population identified as being cancer free so far as probands and first degree

Let this statement include information relatives are concerned versus that population which admits to one or more first degree relatives with cancer. Such a finding would suggest, but of course by no means confirm, that at least for the smoking issue there does not appear to be an important etiologic effect.

2. We may find a significant relationship between smoking and the presence of cancer in the population prone to cancer versus the population free of cancer. Again, such a conclusion might suggest an etiologic role for smoking, but still this would merely indicate an association but in no way would it indict this as a cause-effect relationship. However, the significance of the association would be more strong if the cancers found in the population prone to cancer and showing an excess of smoking, were carcinomas of the lung, oral cavity and pharynx, esophagus, kidney, or urinary bladder, malignant neoplasms which heretofore have been shown to have a greater or lesser degree of statistical association with smoking history. However, even here we would not be certain of this association, since it could well imply that certain personality characteristics, possibly on a familial basis, may be associated with both cigarette smoking and a predisposition to these and other malignant neoplasms.
3. We may find an excess of cigarette smoking habit amongst the patients and their first degree relatives who have been shown to have a negative family history of cancer when compared to the cancer prone group. Such a finding as this might suggest that, at least in this rather large and generally heterogeneous population, cigarette smoking does not appear to play an important role in cancer etiology in this population.
4. Finally, we may find no statistically significant difference in smoking histories between the cancer prone population and the cancer free population. Such a finding as this would suggest that so far as the alleged carcinogenic effect of cigarette smoking is concerned, that it does not appear to play a significant etiologic role in cancer in a population designated as cancer prone versus a population designated as cancer free. This would tend to add strength to the hypothesis that familial factors and/or genetic factors play a more prominent role in carcinogenesis when at least one possible carcinogenic agent, namely cigarette smoking, is critically analyzed. However, we must also be cautious about these and other observations mentioned above because myriad other exogenous factors, i.e., industrial exposures, variations in trace metals in water supply, dietary differences, solar radiation exposure, other agents under study, including asbestos, as well as agents heretofore unidentified so far as carcinogenesis is concerned may be of crucial importance; and therefore the role of host factors will never be fully delineated in man until all possible exogenous carcinogenic agents are critically analyzed in each patient. Of course, such a complete study as this would be impossible to perform in man, particularly since man is a participant in an extremely competitive, variable, and markedly mobile society. On the other hand, a study of the type we are proposing could provide us with further insight into the possible relationship between an agent which has been claimed by many to be an extremely important cause of cancer, namely cigarette smoking, and the relative relationship to host factors in cancer etiology.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

(A). The office facilities of the Department of Preventive Medicine.

(B). We have computer facilities with a 310 computer, available to us under the direction of Rev. Edward Sharp.

(C). Olivetti calculator 270 and a desk computer.

(D). We have a Winnebago (27 foot) mobile camper equipped with facilities for interviews and for examinations when indicated. The interviewing area affords for complete privacy.

11. Additional facilities required:

None are required

12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available):

1003545384

14. First year budget:

A. Salaries (give names or state "to be recruited")
Professional (give % time of investigator(s)
even if no salary requested)

Medical social worker or
Registered nurse with MPH
Biostatistician

% time	Amount
100%	\$13,000
25%	4,000
	<u>17,000</u>

Technical
Secretary

50%	3,000
Total	20,000
Fringe	1,400

Sub-Total for A 21,400

B. Consumable supplies (by major categories)

Stationery, stamps, etc.
Computer supplies

1,000
<u>1,000</u>

Sub-Total for B 2,000

C. Other expenses (itemize)

Travel for interview

3,000

Sub-Total for C 3,000

Running Total of A + B + C 26,400

D. Permanent equipment (itemize)

Sub-Total for D 3,960

E. Indirect costs (15% of A+B+C)

E	3,960
Total request	<u>30,360</u>

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	22,684	2,000	3,000		4,153	31,837
Year 3						

1003545385

16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Hereditary Progressive A-V Conduction Defect	HEW 1 RO1 HL 15903-01	\$35,000	9/1/73 - 8/31/74
Breast Cancer Family Resources	HEW-N01-CB-33901	95,120	7/1/73 - 6/1/74
Genetics of Cardiac Conduction Defects: Family Studies	Nebraska Heart Association	7,158	7/1/73 - 6/31/74
Order of Eagles		10,000	

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Prospective Genetic Studies of Colon and Lung Cancer: Host Environment Considerations	Research Council at Omaha Veterans Administration Hospital	\$14,200	9/1/73 - 8/1/75
Additional Clinical Centers for the Multiple Risk Factor Intervention Trial for the Prevention of Coronary Heart Disease	RFP-NHLI-74-1	289,101	9/1/74 - 8/1/75
Carcinoembryonic Antigen in Two Cancer Prone Families	NIH - Ca 15635-01	100,503	9/1/73 - 8/31/76

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Check payable to _____

Mailing address for checks _____

Principal investigator

Typed Name _____

Signature _____

Date 7/5/73

Telephone _____

Area Code

Number

Extension

Responsible officer of institution

Typed Name Frank Evans

Title Asst. to the Dean for Financial Affairs

Signature _____

Date 9/5/73

Telephone _____

Area Code

Number

Extension

1003545386

APPENDIX

Epidemiologic Background in Support of Hypothesis

Cancer is not uniformly distributed in the population. There are marked variations in cancer incidence at different anatomic sites, in different populations, and in different areas of the world. Numerous etiologic differences account for such variations in cancer incidence, including differences in genotypes,¹ environmental exposures, habit patterns, occupations, socioeconomic status, and educational factors. A comprehensive exploration of cancer etiology involves integrated consideration of all potentially mitigating factors, not only individually, but also collectively as they interact with each other.¹⁻¹⁶

As we studied families with significantly increased incidences of cancer, compared to the general population, we observed significant paucities in occurrences of the cancers in certain branches of these families and in a number of other families, some of which included the kin of spouses of patients in high-incidence families. We concluded that some kindreds may be characterized by unusual resistance or decreased susceptibility to cancer, just as other kindreds may be characterized by unusual cancer-proneness. This observation is not surprising because significant differences in cancer resistance have been well established in inbred strains of a variety of animals.¹⁷⁻¹⁹ Recent studies in tumor immunology have revealed a crucial relationship between an animal's immune defense system and its response to carcinogens as well as to transplanted cancer cells.^{20,21} Stephenson et al.²² have discussed spontaneous regression of tumors in humans in relation to tolerance and/or resistance to malignancy.

We have made extensive observations of two extended families, or kindreds, which show strikingly increased incidences of cancers^{5,6,23} and have compared these findings with those from two other families studied in a similar manner but in whom the incidence of cancer is far less than that found in the general population.²⁴ Thus, we have identified families characterized by unusual proneness

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to cancer (50 percent in some branches) and others which appear to be resistant to cancer (frequency of cancer of 1 percent or less). We believe that genotypic factors are important in determining these differences which have been observed consistently through multiple generations.

Cancer Genetics and Epidemiology

Discussions of the etiology of "cancer", which is a generic term, are largely meaningless. If cancer is to be delineated further, through acquisition of information relevant to the circumstances surrounding onset, then it will be necessary to design studies of specific histologic varieties of cancer. Thus, for example, adenocarcinoma of the colon may occur as a result of excessive irradiation to the abdomen in treatment for another disease such as endometrial carcinoma or abdominal lymphoma; it may occur spontaneously due to unknown etiology without any known mitigating circumstances; it may occur in context with longstanding active ulcerative colitis; it may result from familial polyposis coli, a condition transmitted by an autosomal dominant gene; or it may result from any of several other inherited precancerous disorders.² Malignant neoplasms such as adenocarcinoma of the colon may also congregate in certain families in whom the genetic mechanism may be unclear or may be ascribed to multifactorial inheritance.

Multifactorial genetic transmission has been considered as possibly being etiologic in several diseases with variable age of onset, with incidence increasing with age, and with increased empirical risks to relatives of probands. Multiple genetic factors may be involved in each of several histologic varieties of cancer which tend to congregate on a familial basis. Breast cancer, which shows familial tendencies but does not show simple Mendelian patterns of genetic transmission, may fit such an etiologic classification.¹⁴

1003545388

Cancer resistance may be determined on a multifactorial basis. Therefore, it is reasonable to ask the question, "Are families which show a remarkable freedom from cancer receiving this protection on a basis of multiple-gene-transmitted constitutional characters?" Falconer²⁴ suggests that, when considering diseases which may fall into the category of multifactorial inheritance, one should consider together both genetic and environmental factors which make a particular individual more or less likely to develop a disease such as cancer or diabetes mellitus, and that these should be combined into a single measure which he refers to as the individual's "liability". He reasons that the liabilities of individuals in a population form continuous variables and that the apparent discontinuities between "affected" and "normal" arise from "threshold" differences at some levels of the liabilities. Thus, individuals with liabilities above the threshold are affected, and individuals with liabilities below the threshold are not. The liability of any one individual cannot be measured with precision. On the other hand, however, mean liability may be evaluated for a population or group from the incidence of the disease in that population or group. Thus, liability can in principle be expressed in units on a scale that renders its distribution normal (i.e., fitting a Gaussian or normal curve), and measurements of the mean liability then become the standard deviations on the scale.

According to Falconer²⁴ "The analysis provides an estimate of the correlation between relatives in respect to liability, and this leads, with certain assumptions, to an estimate of the heritability of liability in the population. The heritability is the proportion of the variance of liability that is ascribable to additive genetic variance. This is the nearest to which one may approach, with human data, the degree of genetic determination of liability. The data required for the analysis are the incidence of the disease in the

1003545389

population and the incidence in relatives of affected propositi drawn from the population".

It is reasonable to ask the question, "Are families which show a remarkable

The liability or proneness to a disorder is a function of interacting

freedom from cancer, exposed to this protective environment. The degree of

environmental and genetic factors. These factors operate in differing orders

of magnitude depending upon the individual's specific genetic and environmental

diseases which may fall under the category of multifactorial inheritance. In

circumstances. Thus, when environmental factors are more significant, the

should consider together both genetic and environmental factors which make

relative effects of hereditary factors will be less significant. Since families

particular individual more or less likely to develop cancer. For example,

that are cancer-resistant or cancer-prone may be associated with environments

which are not strikingly different from each other, a major differentiating

variable may be genetic factors.

A heritability determination is an estimate of the proportion of the

total phenotypic variance (i.e., individual differences) that can be attributed

to genetic variation in a single generation of some particular population

under one set of environmental conditions. The heritability of cancer may be

defined as the extent to which variation in individual risk of acquiring can-

cer is due to genetic differences. A disease will show a greater-than-zero

heritability if two or more segregating genetic alleles, which manifest dif-

ferent effects upon predisposition and resistance to the disease, occur on at

least one chromosomal locus. Such a trait may show different heritabilities

in different populations which are characterized by genotypic and/or environ-

mental differences, because the manifestations of any particular gene depend

upon interactions between that gene and the overall genotype as well as with

nongenetic or environmental variables. An individual's predisposition or

resistance to a particular disease process depends upon that individual's genetic

norm or range of reaction and the environmental factors interacting with his

genotype. A genetic factor may be manifested only by appropriate genotype-

environment combinations. A gene's harmfulness or usefulness is determined by

the bearer's environment. Thus, the genetic epidemiologist functions as an

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ecologist seeking significant correlations between a disorder and one or another variable from the great array of environmental influences. One's success in such research is related to the uniqueness of the variable and the directness of its effect, to the frequency of the disorder, and to the ease of diagnosing the disorder.

The cancer epidemiologist must constantly scrutinize all possible hereditary and environmental factors which could be of etiologic importance in the development of cancer. Unfortunately, many studies have concerned themselves only with single carcinogenic factors. Very few attempts have been made to relate nongenetic factors to host factors, e.g., cigarette smoking and family history,²⁵ solar radiation and cancer induction in patients with xeroderma pigmentosum,^{3,4} etc. Few studies have been specifically concerned with cancer resistance in humans.^{20,21}

Rationale

Our group has developed new data pertaining to the distribution of cancer in a relatively sizable population (3,261) of allegedly well patients who come to a mobile cancer detection unit for purposes of cancer screening. This was an unselected population, though it may well harbor a slight bias in that motivational factors may have been important in initiating the visit to the unit. However, we believe that the findings that 47% of the patients coming to the unit showed a negative family history of cancer in first degree relatives, a point confirmed with practically identical statistical results by a colleague, Michael Brennan, M.D. and associates at the Michigan Cancer Foundation in Detroit. We believe that these patients and their families harbor an important potential for studies of potentially significant carcinogenic agents in context with genotypic factors which could be important in determining cancer frequency and distribution in the respective families. Thus, it would seem important

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that carcinogenic agents be critically analyzed in context with host factors.

Studies at the infra-human level have shown repeatedly that a relationship exists between genetic factors and cancer susceptibility, i.e., identified through selective inbreeding studies in a variety of animal populations producing highly inbred strains, and that these host factors are important in controlling the predisposition to cancer; and moreover, they also predispose such animals to the effects of certain carcinogens, and therefore potentiate oncogenicity.

We offer a similar rationale and hypothesis for cancer occurrence in man, namely host factors may be important in determining differential oncogenicity to selected carcinogenic agents.

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13. Publications

1. Lynch, H.T.: Hereditary Factors in Carcinoma. In: Recent Results in Cancer Research. Springer-Verlag, New York, 1967.
that carcinogenic agents be critically analyzed in context with host factors.
2. Lynch, H.T. and Krush, A.J.: Heredity and Adenocarcinoma of the Colon. Stud. Gastroenterology 53:517-527, 1967. It repeatedly has been demonstrated that a relationship exists
3. Lynch, H.T., Anderson, D.E., Krush, A.J., and Mukerjee, D.: Cancer, Heredity, and Genetic Counseling: Xeroderma Pigmentosum. Cancer 20: 1796-1801, 1967. Study of a family of cancer-prone patients with
4. Lynch, H.T., Anderson, D.E., Smith, J.L. Jr., Howell, J.B., and Krush, A.J.: Xeroderma Pigmentosum, Malignant Melanoma, and Congenital Ichthyosis: A Family Study. Arch. Dermatol. 96:625-635, 1967.
5. Lynch, H.T. and Krush, A.J.: Cancer Family "G" Revisited: 1895-1970. Cancer 27:1505-1511, 1971.
6. Lynch, H.T., Shaw, M.M., Magnuson, C.W. and Krush, A.J.: Hereditary Factors in Cancer: Study of Two Large Midwestern Kindreds. Arch. Intern. Med. 117: 206-212, 1966.
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9. Beolchini, P.E., Cresseri, A., Gianferrari, L., Malcovati, P., and Morganti, G.: Ricerche Genetiche Sulle Neoplasie dell'Utero (Parte II). Acta Genet. Med. Gemellol. 6:59-84, 1957.
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12. Lynch, H.T., Krush, A.J., Larsen, A.L., and Magnuson, C.W.: Endometrial Carcinoma: Multiple Primary Malignancies, Constitutional Factors, and Heredity. Amer. J. Med. Sci. 252:381-390, 1966.
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15. Lynch, H.T. and Krush, A.J.: Carcinoma of the Breast and Ovary in Three Families. Surg. Gynec. Obstet. 133:644-648, 1971.

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16. Lynch, H.T., Krush, A.J., Harlan, W.L. and Sharp, E.A.: Association of Breast Cancer, Soft Tissue Sarcoma, Leukemia, and Brain Tumors in Breast Cancer Families. Amer. Surg. **39**:199-206, 1973.
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17. Jacobson, J. et al. TABLE 1. Distribution of Participants in Omaha and Detroit to the

Number of Cancers in the Family

Group I Group II

Category Participants Number Percent Number Percent

No History of Cancer 1520 46.6 470 46.6

History of a Single Cancer 1082 33.2 296 29.3

History of Multiple Cancers 659 20.2 243 24.1

Total 3261 100.0 1009 100.0

Category Participants Number Percent Number Percent

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TABLE 2

**DISTRIBUTION OF BREAST AND OTHER CANCERS IN GRANDMOTHERS,
MOTHERS AND AUNTS OF PARTICIPANTS UNDER 45 YEARS**

Group No.	Relation to Participant	No. of Breast Cancers	No. of Other Cancers	Total Cancers	% of Breast Cancer
Group I	Grandmothers	37	178	215	17.2
	Mothers	45	90	135	33.3
	Aunts	58	154	212	27.4
Group II	Grandmothers	66	178	244	27.0
	Mothers	44	52	96	45.8
	Aunts	74	135	209	35.4

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TABLE 3

DISTRIBUTION OF BREAST AND OTHER CANCERSIN FEMALE RELATIVES OF PARTICIPANTS45 YEARS OF AGE AND OVER

	<u>Breast Cancer</u>	<u>Other Cancer</u>	<u>Total Cancer</u>	<u>% of Breast Cancer</u>
Mothers	80	377	457	17.5
Sisters	100	279	379	26.4
Female probands	42	88	130	32.3
Proband's daughters	6	44	50	12.0

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TABLE 4

THE OCCURRENCE OF BREAST AND OTHER CANCERS IN LINEAGES OF PARTICIPANTS

	Group I		Group II	
	No. of Lineages Involved 1244		No. of Lineages Involved 2044	
	Number	Percent	Number	Percent
1 breast cancer and no other cancer	59	4.7	84	4.1
1 breast cancer + 1 other cancer	35	2.8	58	2.8
1 breast cancer + 2 other cancers	12	1.0	5	0.2
1 breast cancer + 3 other cancers	2	0.2	3	0.1
2 breast cancers and no other cancers	7	0.6	6	0.3
2 breast cancers + 1 other cancer	1	0.1	7	0.3
2 breast cancers + 2 other cancers	2	0.2	3	0.1

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TABLE 5

Total Number Screened in Each Location

Town	Total	Male	Female
Macy	280	78	202
Winnebago	197	66	131
Lynch	670	262	408
Bassett	466	131	335
South Sioux City	279	84	195
Tekamah	680	196	484
Holdrege	241	62	179
Omaha	220	66	154

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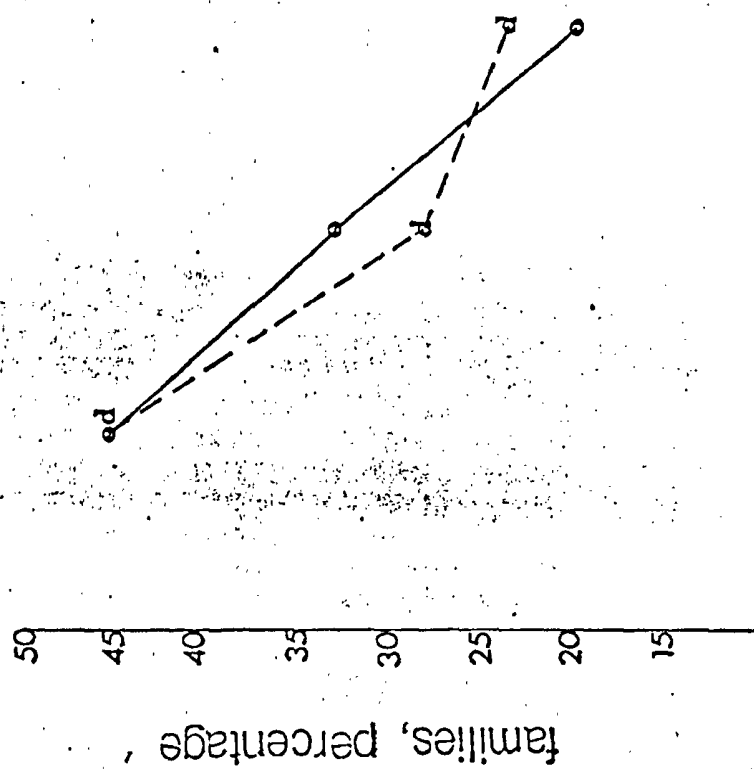
Figure 1

The history of cancer of all sites in
families studied in Groups I and II

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HISTORY OF CANCER OF ALL SITES

--- Detroit study
— Omaha study



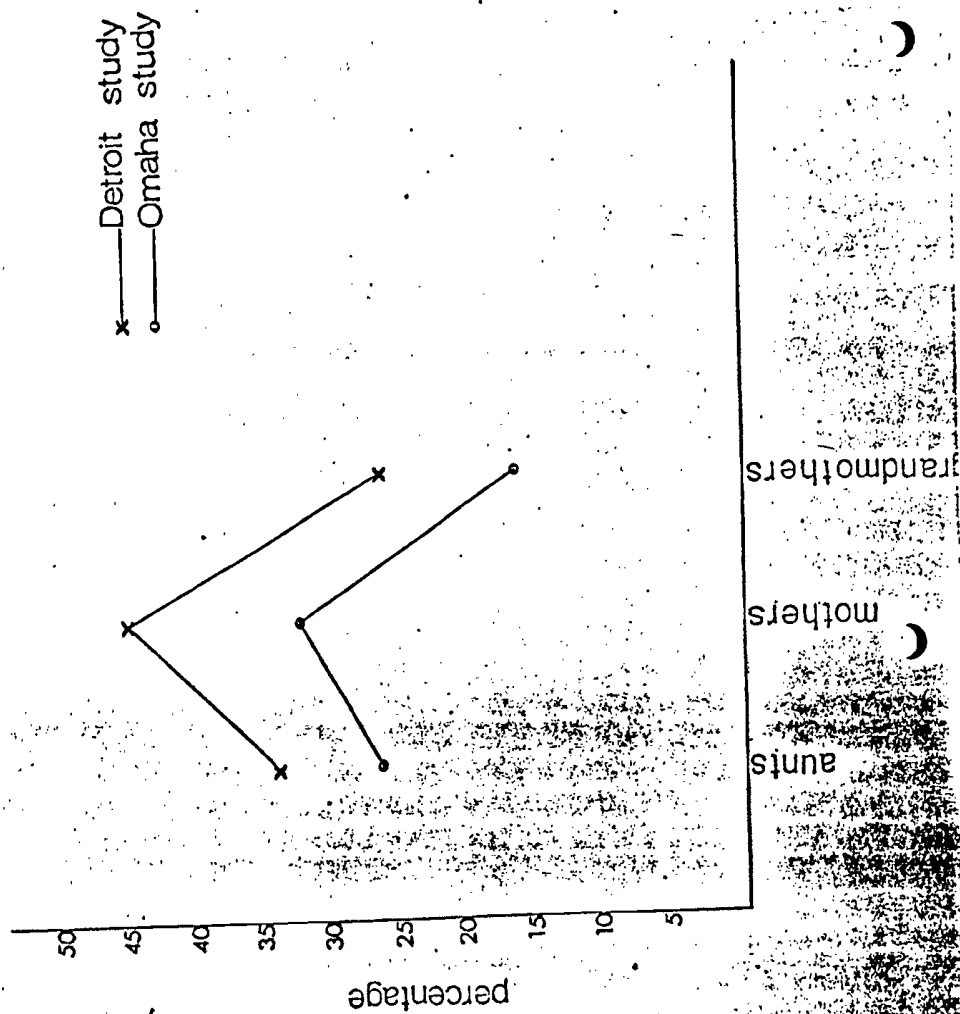
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• Figure 2

The distribution of breast and other cancers
in Grandmothers, mothers and aunts of partici-
pants under the age of 45 years in Groups I
and II

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Distribution of Breast Cancers



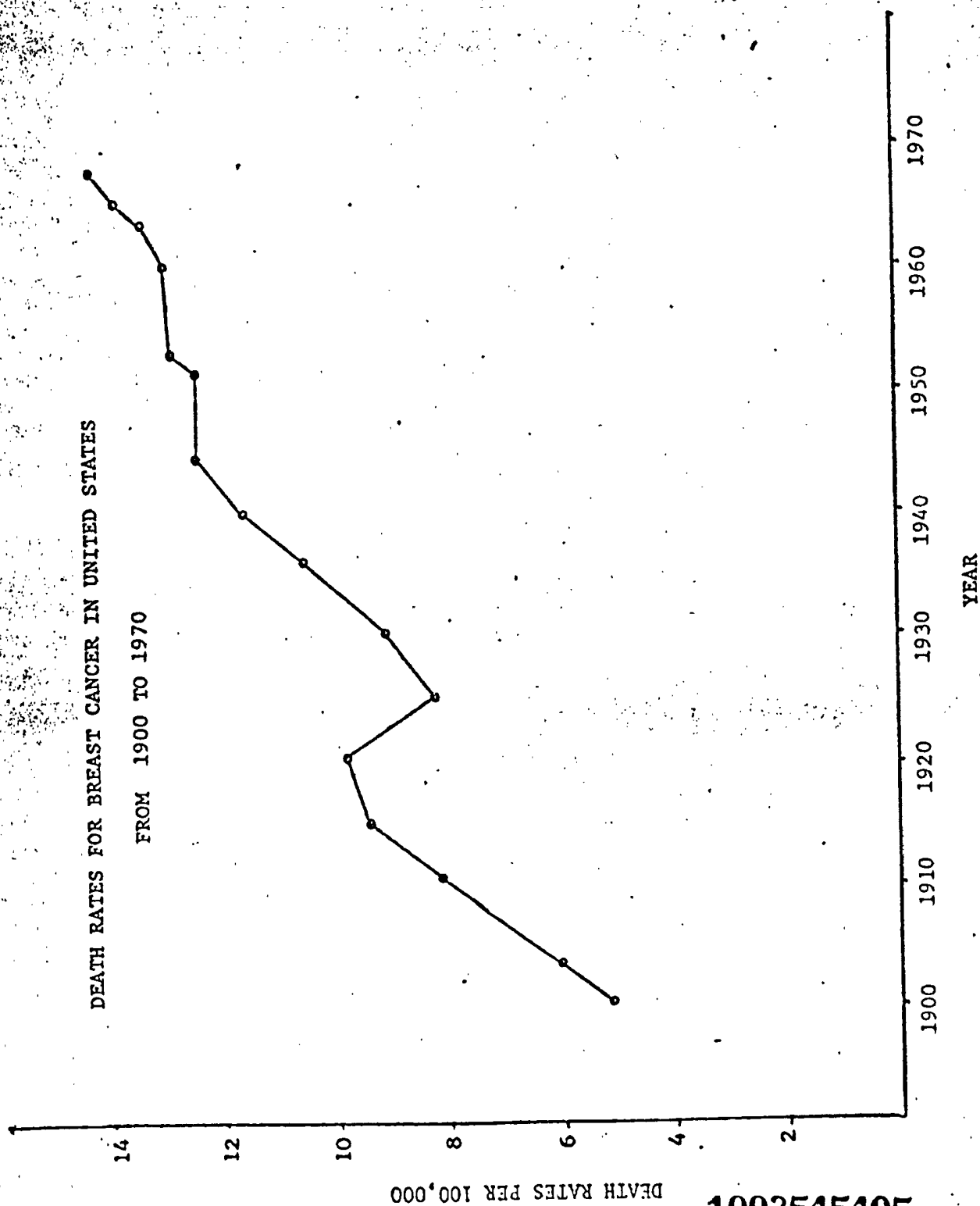
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Figure 3

Death rates per 100,000 of breast cancer
in United States from the years 1900-1970

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DEATH RATES FOR BREAST CANCER IN UNITED STATES
FROM 1900 TO 1970



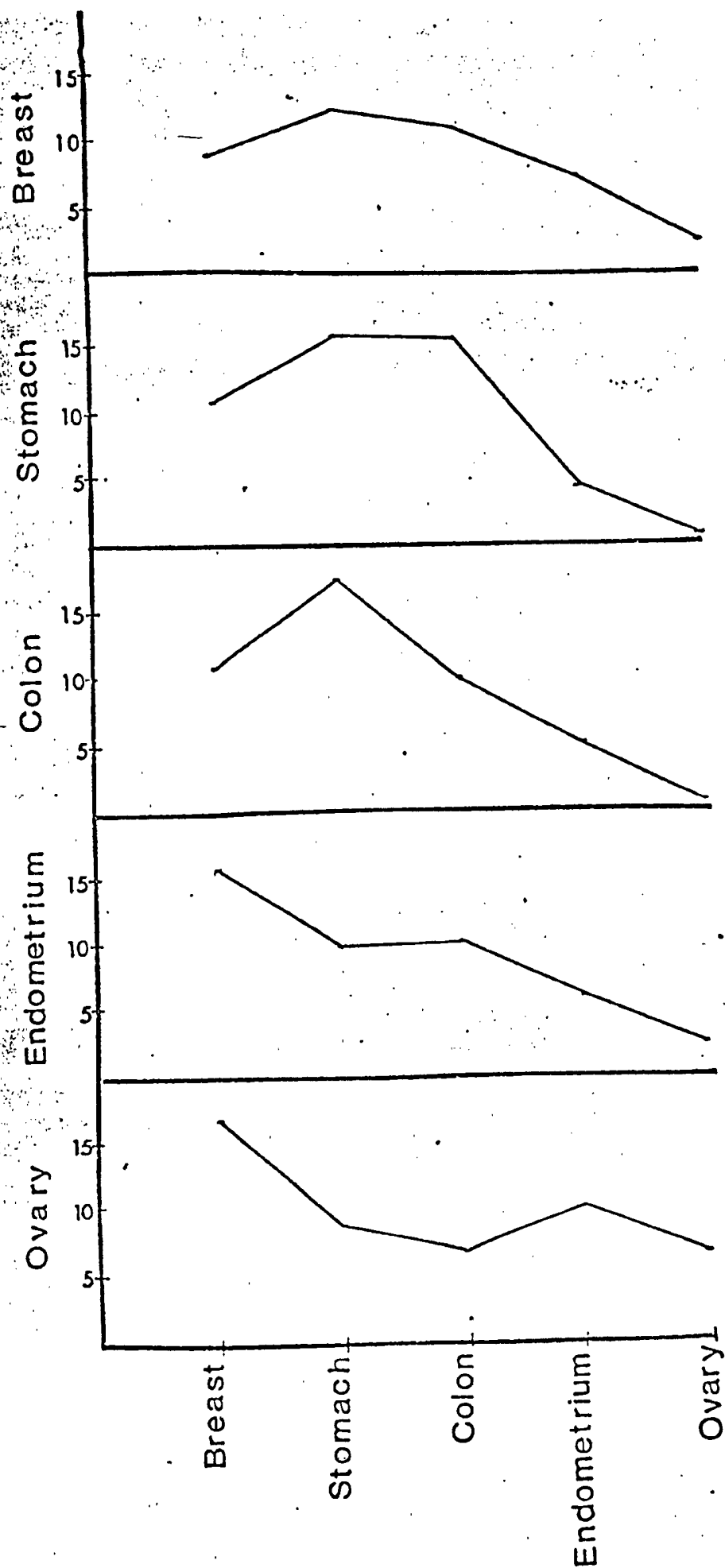
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Figure 4

A cross comparison of associations of
carcinoma of the Breast, Stomach, Colon,
Endometrium, and Ovary of families studied

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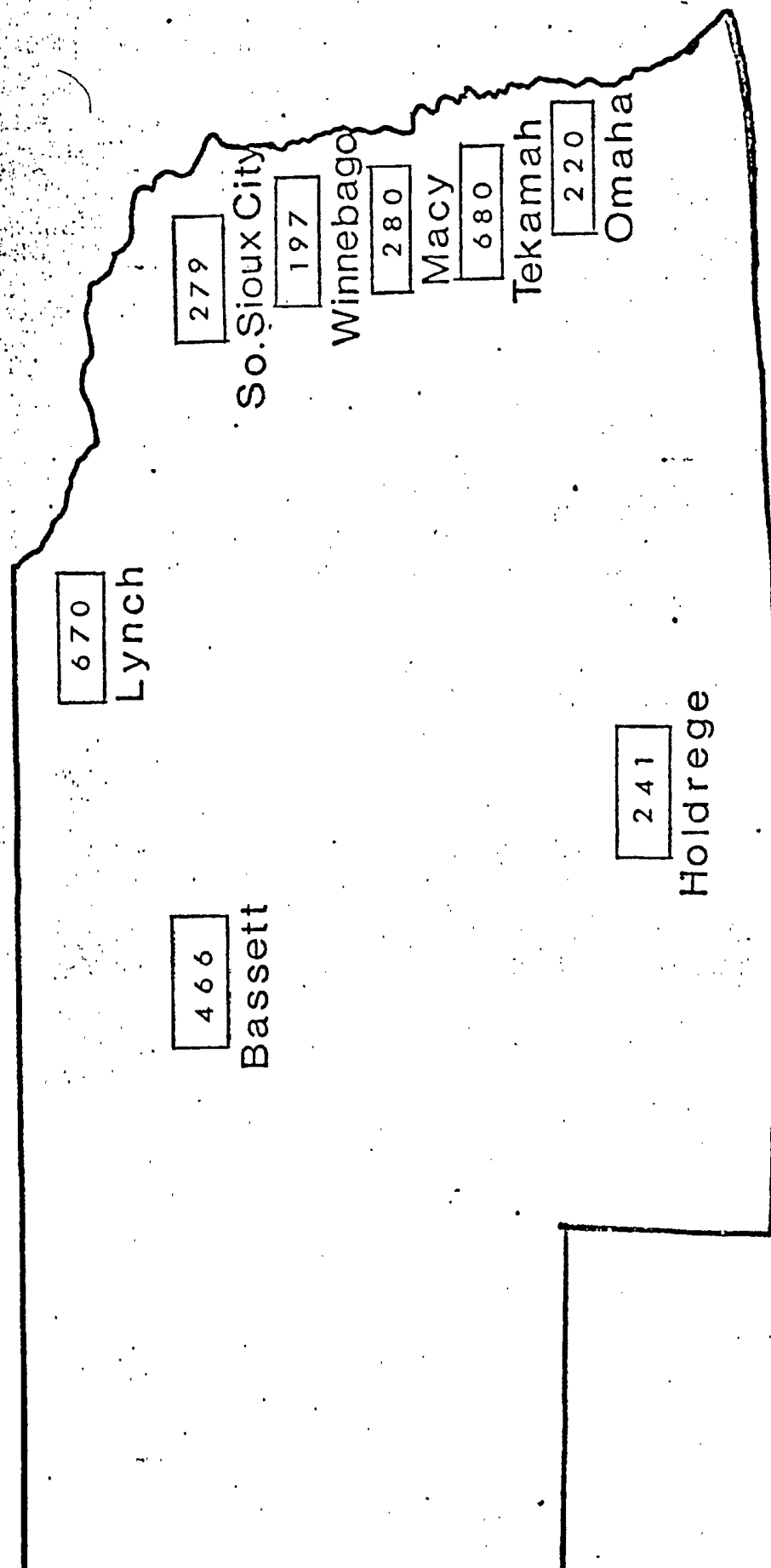
PERCENT ASSOCIATION



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FIGURE 5

DISTRIBUTION OF POPULATION IN THE STATE OF NEBRASKA



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SECTION II - PRIVILEGED COMMUNICATION

BIOGRAPHICAL SKETCH

(Give the following information for all professional personnel listed on page 3, beginning with the Principal Investigator. Use continuation pages and follow the same general format for each person.)

NAME Henry T. Lynch	TITLE Principal Investigator	BIRTHDATE (Mo., Day, Yr.) R
PLACE OF BIRTH (City, State, Country)	PRESENT NATIONALITY (If non-U.S. citizen, indicate kind of visa and expiration date)	SEX <input checked="" type="checkbox"/> Male <input type="checkbox"/> Female

REDACTED

EDUCATION (Begin with baccalaureate training and include postdoctoral)

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	SCIENTIFIC FIELD
University of Oklahoma, Norman, Oklahoma	B.S.	R	Psychology
Denver University, Denver, Colorado	M.A.	R	Psychology
University Texas, Med. Branch, Galveston	M.D.	R	Medicine

HONORS Psy Chi, Honorary Society in Psychology, 1950. Society of Sigma Xi, 1959.
Billings Silver Medal for Exhibit, "Heredity and Carcinoma: A Study of Cancer Families"
(Presented to A.M.A., Chicago, June, 1966.)

MAJOR RESEARCH INTEREST Cancer and Medical Genetics	ROLE IN PROPOSED PROJECT Principal Investigator
--	--

RESEARCH SUPPORT (See instructions)

- Received NIH Training Grant: "Morbidity and Mortality from Hematopoietic and Solid Malignant Tumors and Their Possible Relationship to Dermographic and Ecologic Factors in a Community." #5A07 AH 00210-05 3/1/73-2/28/74 1972 - \$5840 Total for 5 years = \$29,200, Percent of effort = 10%.
- Regional Med. Program (NIH) for Mobile Cancer Screening Unit, 1972-1973, \$459,950 Percent of Effort = 10%.
- Hereditary Factors in Adenocarcinoma of Colon--approved by, but not funded by Omaha VA Hospital (Supported by Damon Runyon Memorial Ca. Fund, 1969-1970. Percent of Effort = 10%.
- Biological Studies of the Ca. Family Syndrome. NIH approved, waiting for funding, 4/1973.

RESEARCH AND/OR PROFESSIONAL EXPERIENCE (Starting with present position, list training and experience relevant to area of project. List all or most representative publications. Do not exceed 3 pages for each individual.)

1967 to Present: Professor and Chairman, Department of Preventive Medicine and Public Health and Assistant Professor of Medicine, Creighton University School of Medicine, Omaha, Nebraska; 1966-1967: Assistant Professor of Biology, Assistant Internist, Department of Medicine (part-time), the University of Texas, M.D. Anderson Hospital and Tumor Institute, Houston, Texas; 1964-1966: Instructor, Internal Medicine; Senior Cancer Trainee, U.S.P.H.S., in Cancer and Allied Disease, Omaha, Nebraska; 1962-1965: Medical genetics consultant and lecturer, Department of Orthodontics, Lincoln, Nebraska; 1962-1964: Lecturer in Human Genetics, University of Nebraska College of Medicine, Omaha, Nebraska.

REPRESENTATIVE PUBLICATIONS

- Lynch, H.T., Shaw, M.W., Magnuson, C.W., Larsen, A.L., and Krush, A.J.: Hereditary Factors in Cancer: Study of Two Large Midwestern Kindreds, Arch. Intern. Med. 117:206-212, 1966.
- Lynch, H.T., Lemon, H.J., and Krush, A.J.: A Note on "Cancer-Susceptible" and "Cancer-Resistant" Genotypes: Implications for Cancer Detection and Research, Nebraska Med. J. 51:209-211, 1966.

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2 Lynch

REPRESENTATIVE PUBLICATIONS (Con't.)

3. Lynch, H.T., Anderson, D.E., Krush, A.J., and Larsen, A.L.: Heredity and Carcinoma, Ann. N. Y. Acad. Sci. **115**:793-800, (December) 1968.
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11. Lynch, H.T., Guirgis, H.A., Swartz, M.W., Lynch, J.S., Krush, A.J., and Kaplan, A.R.: Genetics of Colon Cancer, Arch. Surg., In Press.
12. Lynch, H.T., Krush, A.J., and Kaplan, A.R.: Cancer Frequency Variations Among and Within Families, Acta Genet. Med. Gemellol. **21**:53-65, 1972.

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1003545410

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

November 9, 1973

To: Committee comprising Huebner, Jacobson, Sommers, Meier, and
W.U.G., R.C.H., F.W.N.

From: J.H.K.

Subject: Genetic Study of AHH and its Relationship to Lung-Colonic Cancer
No. 941 Grant Application Addendum, Dr. Henry Lynch, Creighton
University

At staff request Dr. Henry Lynch has developed a protocol to include an analysis for arylhydrocarbon hydroxylase (AHH) in his proposed study of human high risk (and low risk) families. Recent papers from M. D. Anderson (Dr. C. Shaw) have pointed to a high association between AHH inducibility and lung cancer. Dr. Shaw also (unpublished data) states a high correlation exists between AHH induction and colonic cancer. The attached letter and supplement to Grant Application No. 941 proposes to obtain the genetic information with regard to AHH induction in a high susceptibility population. The enlarged program would allow us to begin to look at smoking history and AHH inducibility together and separately, as well as other possible susceptibility factors in the etiology of lung cancer.

This program should be considered in conjunction with studies of the lung cancer population at M. D. Anderson, possible twin studies, and epidemiological studies of high lung cancer populations in the Los Angeles basin near oil refineries (allowing us to begin to define polycyclic aromatic hydrocarbons and air pollution involvement in AHH induction, and any synergistic effects of smoking and other factors in the etiology of lung cancer.

Meetings are being held between the various groups to define and improve the testing system (more sensitivity needed) and to begin to coordinate the questionnaires to be used in the epidemiology studies. Part of the latter item and the mechanism for uniform testing should be considered at the Twin Conference in Miami in December in order to avoid confusion later on. The test system to be used is initially important, and must be standardized in order to produce unequivocal results in different laboratories. An additional effort must be spent in this regard within the next six months in order to make large population screening studies possible by late next spring. One grant has been given to M. D. Anderson to improve their test system (currently only 15 persons can be tested per week). Possibly another small contract should be let by CTR to clean up the system and assure we will be able to get the population studies underway as soon as possible. It would be desirable to hold a small conference including Drs. Gelboin, Nebert, Conney, Kellerman and Kouri in early spring to iron out final details of methods in the test system.

This program to define a population at risk and the relationship of smoking and disease occurrence in this population is of high relevance. These programs should be considered in the development of budget for the coming year, with the consideration of an adequate supplemental budget if necessary to carry out the development of programs such as mentioned above.

JHK:wg

J.H.K.

1003545411



CREIGHTON UNIVERSITY

OMAHA, NEBRASKA 68178

SCHOOL OF MEDICINE
DEPARTMENT OF PREVENTIVE MEDICINE
AND PUBLIC HEALTH

November 7, 1973

Dr. John H. Kreisher
The Council for Tobacco Research--
U.S.A., Inc.
110 East 59th Street
New York, New York 10022

Dear John:

I respectfully enclose our application for research grant entitled:
"Aryl Hydrocarbon Hydroxylase (AHH): Cancer Genetics" for consideration
by the Council.

I am sorry about the delay which I encountered in getting this material
in your possession. You will note that this proposal represents a natural
extension of our prior research application designed for the study of
variations in cancer susceptibility in a large normal population. Since
talking with you on our recent visit to Dr. Shaw's laboratories at the MD
Anderson Hospital in Houston, we have actively put together the necessary
talent for these studies and I am pleased to report that we now have the
capacity, and in fact, are doing a limited number of these determinations
on certain high risk cancer patients.

I talked with Dick Kouri a few days ago pertaining to expansion of
his own activities which would include our own collaboration with him for
AHH studies of high risk cancer patients. It would be of great value if
this eventually comes to fruition.

We are very excited about the prospects of being able to expand our
activities in the area of AHH determinations in the variety of cancer
susceptible clinical problems. I am impressed with the interest that is
being shown about this problem nationally.

Best of luck to you, John, and I hope that our paths will cross again
soon. Possibly at the meetings in Miami.

Sincerely,

Henry T. Lynch, M.D.
Professor and Chairman
Department of Preventive Medicine
& Public Health

1003545412

HTL/sah

Comm.

EPIDEMIOLOGY

#941-Pt. II

Dr. Gardner
Dr. Huebner
Dr. Jacobson

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

110 EAST 50TH STREET
NEW YORK, N. Y. 10022
(212) 421-8585

NOV 9 1973

Application for Research Grant
(Use extra pages as needed)

Date:

1. Principal Investigator (give title and degrees):

Henry T. Lynch, M.D., Professor and Chairman, Dept. of Preventive Med. and Public Health
Ibert C. Wells, Ph.D., Professor and Chairman, Dept. of Biological Chemistry
Hoda Guirgis, Ph.D., Assistant Professor, Dept. of Preventive Med. and Public Health

2. Institution & address:

Creighton University School of Medicine
2500 California Street
Omaha, Nebraska 68178

3. Department(s) where research will be done or collaboration provided:

Department of Preventive Medicine and Public Health
Department of Biological Chemistry

4. Short title of study:

Aryl hydrocarbon hydroxylase (AHH): Cancer genetics

5. Proposed starting date: January 1, 1974

6. Estimated time to complete: 3 years

7. Brief description of specific research aims:

Inducibility of aryl hydrocarbon hydroxylase (AHH) will be measured in lymphoblasts from patients from low and high risk cancer prone families in order to determine familial patterns of AHH induction susceptibilities (low, medium, and high). Possible associations between cancer risk and the inducibility of AHH will be correlated with specific histologic varieties of cancer and their genetic modes of transmission. Intensive tumor and genealogic documentation will permit critical appraisal of the significance of AHH findings.

The association of AHH induction susceptibility with other factors, e.g. smoking history, drug consumption, environmental exposures to carcinogens including occupational carcinogens and cancer history will be studied.

As a continuation of this study, it will be of interest to investigate as possible markers other enzymes that are simultaneously induced in lymphoblasts by various carcinogens.

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2.
8. Brief statement of working hypothesis:

Cancer prone and cancer free families, when appraised with a high degree of validity and reliability through histologic verification of cancer and precise genealogy, provide valuable resource material in the quest for biochemical markers indicating cancer risk. AHH, having shown increased inducibility in patients with carcinoma of the lung, and possibly in adenocarcinoma of the colon, merits testing in well-defined human clinical cancer genetic problems. If it could be demonstrated that patients at high genetic risk for cancer also have concomitant high susceptibility for AHH inducibility (the reciprocal for low cancer genetic risk patients), then we would have a potentially valuable experimental test for the study of carcinogenesis in man. This would also provide additional diagnostic information, particularly when coupled with cancer genetic risk, and could be utilized in cancer control programs. In summary, we would suspect that the genetic aspect of carcinogenesis might be concerned with the inducibility of AHH and/or other mixed function oxidases; chemical carcinogenesis will then depend upon the conversion of potential carcinogens into active carcinogens, by these mixed function oxidases. Our hypothesis will, therefore, be tested in clinical models noteworthy for genetic susceptibility or resistance to cancer.

9. Details of experimental design and procedures (append extra pages as necessary) (See Appendix for literature review)

Aryl hydrocarbon hydroxylase (AHH) inducibility will be determined in lymphoblasts by measuring AHH activities of cells exposed to 3-methylcholanthrene and dibenzanthracene and comparing these activities with those determined in control cells not exposed to these materials. The procedure to be employed is a modification of the fluorometric procedure of Kellerman *et al* (18). Fifteen to 20 ml. of heparinized blood will be collected from each patient and total lymphocytes will be separated using 4% dextran solution. These cells will then be incubated in culture media containing phytohemagglutinin, pokeweed mitogen, heparin and fetal calf serum at 37°C. for 72 hours. 3-methylcholanthrene and dibenzanthracene will be added separately to duplicate cultures and incubation will be continued for another 24 hours. Culture media will be removed from all cell cultures and replaced with buffer medium containing NADH, NADPH and 3,4-benzpyrene. Incubation will be continued for 35 minutes and the enzymatic reaction then will be stopped by the addition of 25% acetone in hexane which will also extract the reaction product (3-hydroxybenzpyrene) from the buffer solution. The hexane solutions will then be extracted with 1 N NaOH which selectively removes the reaction product from the NaOH insoluble benzpyrene. The amount of reaction product in each NaOH extract will be determined fluorometrically using an Aminco-Bowman spectrofluorometer with excitation at 396 nm. and emission at 522 nm. Amounts of 3-hydroxybenzpyrene formed will be expressed for comparison purposes, per 3×10^6 cells per 35 minutes. AHH inducibility will then be expressed as the ratio of the amount of 3-hydroxybenzpyrene formed in cells exposed to 3-methylcholanthrene or dibenzanthracene to the amount of 3-hydroxybenzpyrene formed by identical cells not exposed to these hydrocarbons.

AHH inducibilities (as well as AHH levels) will be determined on probands and selected relatives so that genetic cancer risk, determined by pedigree analysis, will provide a testable experimental parameter. The following groups will be studied: Group A, 200 probands having no history of cancer, their spouses and their children; Group B, 100 probands having one first degree relative with cancer, their spouses and their children; Group C, 100 probands having 2 or more first degree relatives with cancer, their spouses and their children; and Group D, 200 relatives from cancer-prone and 200 relatives from cancer-free lines of families with the cancer family syndrome. These groups will provide a population sample of about 2000 individuals. Blood samples will be collected in the field and the samples will be transported to Omaha and processed within 12 hours of collection.

Standard statistical methods will be used to test significance of correlations.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

A laboratory with the usual equipment for biochemical research such as pH meters, colorimeters, centrifuges, etc. is available together with the following special equipment: Beckman quartz spectrophotometer, Amino-Bowman spectrofluorometer with photo multiplier microphotometer and strip chart recorder, refrigerators, deep freezers, Amino refrigerated bath, Virtis freeze-dry apparatus, refrigerated low-speed centrifuge (International HR-1), Spinco preparative ultracentrifuges (L and L2-65), Spinco analytical ultracentrifuge (Model E) with schlieren and ultra violet optics, walk-in refrigerated room, Dubnoff incubator, Warburg apparatus, autoclave, a variety of chromatography equipment including that for paper, columns, thin-layer and gas-liquid, LKB fraction collector, a variety of electrophoresis equipment including that for paper, cellulose strip, starch gel, agarose and disc (both analytical and preparative) using polyacrylamide gels, and LKB immuno-electrophoresis equipment. A Beckman amino acid analyzer (Model 120 B) is also available together with the ancillary equipment necessary for the amino acid analysis of proteins. In addition, there is an autoanalyzer (Technicon) for use in the assay of column eluates for peptides, and an isotope laboratory which contains the usual facilities for handling labeled compounds together with the following special instruments: scaling unit, automatic sample changer and windowless flow counter (Q-gas counter), strip counters (Nuclear-Chicago and Packard), radioactivity survey meter and three-channel liquid scintillation spectrometer with automatic background subtract and calculator (Nuclear-Chicago). A complete facility for tissue culture is available including incubators, aseptic work areas, etc. Finally there are available well-kept and supervised animal quarters. A 27 ft. Winnebago motor home modified to include necessary laboratory facilities (centrifuges, etc.) will be used for sample collection.

11. Additional facilities required: None

12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).
Reprints not available.

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CURRICULUM VITAE

NAME: Henry T. Lynch

S.S. NUMBER:

REDACTED

PLACE AND DATE OF BIRTH:

REDACTED

PRESENT ADDRESS:

Department of Preventive Medicine
and Public Health
The Creighton University
School of Medicine
Omaha, Nebraska 68178 -

MARITAL STATUS:

REDACTED

EDUCATION:

B.S. *R*M.A. *R***REDACTED**

University of Oklahoma, Norman
Denver University, Denver
University of Texas, Austin
Work toward Ph.D. in Human Genetics
Major field: Human Genetics
Minor field: Biochemistry
Psychology

Course work completed. Dissertation
was in progress on admission to Medical
School

M.D. *R*

University of Texas Medical Branch, Galveston
St. Mary's Hospital, Evansville, Indiana
Rotating Internship completed

REDACTED**REDACTED**

University of Nebraska College of Medicine
Residency in Internal Medicine completed
A "Short Course in Medical Genetics," supported
by the National Foundation; Coordinated by
Dr. Victor McKusick, Bar Harbor, Maine,
August 3-14

REDACTED

Senior clinical cancer trainee, U.S.P.H.S.,
Epplcy Institute for Research in Cancer and
Allied Diseases, University of Nebraska College
of Medicine, Omaha, Nebraska

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- 1962-65 Medical Genetics Consultant and Lecturer, Department of Orthodontics, Dr. Sam Weinstein, Chairman, University of Nebraska College of Dentistry, Lincoln, Nebraska.
- 1962-64 Lecturer in Human Genetics, Graduate and Undergraduate students, Department of Zoology, Dr. Dwight Miller, Chairman, University of Nebraska, Lincoln, Nebraska.
- 1964-66 Instructor, Internal Medicine; Senior Cancer Trainee, U.S.P.H.S., University of Nebraska College of Medicine and Eppley Institute for Research in Cancer and Allied Diseases, Henry M. Lemon, M.D., Director.
- 6/1/66-10/67 Assistant Professor of Biology, Department of Biology, Assistant Internist, Department of Medicine, Section of Human Genetics, the University of Texas M. D. Anderson Hospital and Tumor Institute, Houston, Texas.
- 10/67 Associate Professor and Chairman, Department of Preventive Medicine and Public Health, The Creighton University School of Medicine, Omaha, Nebraska.
- 6/1/68 Assistant Professor, Department of Medicine, Creighton University School of Medicine.
- 9/1/70 Professor & Chairman, Dept. of Preventive Medicine and Public Health, The Creighton University School of Medicine, Omaha, Nebraska.
- 1/72 Subcommittee on Epidemiology of the Breast Cancer Task Force National Cancer Institute, Bethesda, Maryland

MEMBERSHIP IN SCIENTIFIC SOCIETIES:

REDACTED

REDACTED

REDACTED

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Personal Publications (five most recent)

Lynch, H.T., Krush, A.J., Lemon, H.M., Kaplan, A.R., Condit, P.T., and Bottomley, R.H.: Tumor Variations in Families with Breast Cancer, J.A.M.A. 222:1631-1635, 1972.

Lynch, H.T., Krush, A.J., and Kaplan, A.R.: Cancer Frequency Variations Among and Within Families, Acta Genet. Med. Gemellol. 21:53-65, 1972.

Lynch, H.T., Guirgis, H.A., Swartz, M.W., Lynch, J.S., Krush, A.J., and Kaplan, A.R.: Genetics and Colon Cancer, Arch. Surg. 106:669-675, 1973.

Lynch, H.T., Krush, A.J., Harlan, W.L., and Sharp, E.A.: Association of Soft Tissue Sarcoma, Leukemia, and Brain Tumors in Families Affected with Breast Cancer, Amer. Surg. 39:199-206, 1973.

Lynch, H.T., Lynch, J., and Kraft, C.: A New Approach to Cancer Screening and Education, Geriatrics 28:152-157, 1973.

Lynch, H.T., Kaplan, A.R., Moorhouse, A., Krush, A.J., and Clifford, G.: Dermatoglyphic Peculiarities in Members of a High-Cancer-Risk Kindred, Oncology, in press.

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A. Co-Principal Investigator: Ibert C. Wells, Ph. D.

Biographical Sketch:

Male -

REDACTED

REDACTED

Education:

A. B. (chemistry and mathematics) Central Methodist College, Fayette, Missouri R Ph. D. (biochemistry under E. A. Doisy) St. Louis University, St. Louis, Missouri R Postdoctoral fellow (NRC) at the California Institute of Technology, R , under Linus Pauling. Research was concerned with physiochemical study of sickle cell hemoglobin (Hb-S).

Professional Experience:

Creighton University School of Medicine, Omaha, Nebraska,

Professor of Biochemistry and Chairman, Department of Biochemistry, 1961- Research has been concerned with the metabolism and metabolic effects of choline, and serum enzymes especially lecithin: cholesterol acyl-transferase and atherogenesis.

State University of New York Upstate Medical Center,

Syracuse, New York. Instructor of Biochemistry, Department of Biochemistry, 1950-52; Assistant Professor, 1952-56; Associate Professor, 1956-61. Research was concerned with synthesis of antibiotics produced by Pseudomonas aeruginosa, biosynthesis of cholesterol, and studies of metabolic efforts of choline antimetabolites.

Society Memberships:

REDACTED

REDACTED

Honors and Awards:

Co-winner, Commercial Solvents Corp. Award in Antibiotics (administered by Am. Soc. Bacteriologists), 1952. Listed in Who's Who in America, 1968 -.

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Personal Publications (five most recent)

Wells, I. C., "Hemorrhagic kidney degeneration in choline deficiency", Federation Proceedings 30, 151 (1971).

Wells, I. C., "Release of intracellular enzymes in serum", Canad. J. Biochem. 47, 347 (1969).

Wells, I. C. and Rongone, E. L., "Dietary cholesterol and serum cholesterol esterifying activity in rabbits", Proc. Soc. Exp. Biol. and Med. 127, 1006 (1968).

Wells, I. C. and Hogan, J. M., "Effects of dietary deficiencies of lipotropic factors on plasma cholesterol esterification and tissue cholesterol in rats", J. Nutrition 95, 55 (1968).

Wells, I. C. and Krajewski, J. P., "Hormonal influences on choline concentrations in rat tissues", Endocrinology 82, 693 (1968).

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14. First year budget:

A. Salaries (give names or state "to be recruited")
Professional (give % time of investigator(s)
even if no salary requested)

	% time	Amount
Medical Technologist	100%	\$ 8,000
Junior Technician	100%	6,000
TOTAL		14,000

Technical

Fringe 1,120

Sub-Total for A 15,120

B. Consumable supplies (by major categories)

Blood culture tubes	1,500
Conical centrifuge tubes	750
Disposable microfilters	500
Culture media	2,000
Chemicals	2,000
Other supplies (pipettes, flasks, cuvettes —)	1,500

Sub-Total for B 8,250

C. Other expenses (itemize)

Travel for sample collection	1,500
Maintenance of equipment	500

Sub-Total for C 2,000

Running Total of A + B + C 25,450

D. Permanent equipment (itemize)

Sub-Total for D 3,817

E. Indirect costs (15% of A+B+C)

Total request 29,267

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	15,876	8,500	2,000		3,956	30,332
Year 3	16,669	8,800	2,000		4,120	31,589

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16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE			
Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Hereditary Progressive A-V Conduction Defect	HEW 1 R01 HL 15903-01	\$35,000	9/1/73 - 8/31/74
Breast Cancer Family Resources	HEW-N01-CB-33901	95,120	7/1/73 - 6/1/74
Genetics of Cardiac Conduction Defects: Family Studies	Nebraska Heart Association	7,158	7/1/73 - 6/31/74
Order of Eagles	Nebr. Tuberculosis and Respiratory Disease Ass'n.	10,000	7/1/73 - 6/31/74
Isolation and characterization of SRS-A	Nebr. Heart Ass'n.	2,500	7/1/73 - 6/31/74
Lysolecithin in atherogenesis		4,130	

PENDING OR PLANNED			
Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Prospective Genetic Studies of Colon and Lung Cancer: Host Environment Considerations	Research Council at Omaha Veterans Administration Hospital	\$14,200	9/1/73 - 8/1/75
Additional Clinical Centers for the Multiple Risk Factor Intervention Trial for the Prevention of Coronary Heart Disease	RFP-NHLI-74-1	289,101	9/1/74 - 8/1/75
Carcinoembryonic Antigen in Two Cancer Prone Families	NIH - Ca 15635-01	100,503	9/1/73 - 8/31/76

is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Checks payable to

Creighton University

Mailing address for checks

2500 California

Omaha, Nebraska 68178

Principal investigator

Typed Name Henry T. Lynch, M.D.

Signature *[Signature]* Date 11/7/73Telephone *[REDACTED]* Extension

Responsible officer of institution

Typed Name LeRoy Kozény

Title Controller

Signature *[Signature]* Date 11/7/73Telephone *[REDACTED]* Area Code Number Extension

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Literature Review

According to presently accepted concepts, the carcinogenic polycyclic aromatic hydrocarbons must be metabolized by certain mixed-function oxidases to reactive intermediates to elicit cell transformation, mutagenicity and cytotoxicity (1).

Aryl hydrocarbon hydroxylase (AHH) is one of these mixed function oxidases and occurs in the microsomal fraction of most tissues of the mouse and other experimental animals investigated (2-4) and probably in most tissues of man (5-8). It is an inducible enzyme since its activity is increased after the administration to animals of a number of different agents, including polycyclic hydrocarbons, drugs, steroids, insecticides and various other substances (9).

Recently, Kouri, et al. (10) have reported a relationship between the inducibility of AHH in mice and the susceptibility to 3-methylcholanthrene induced tumors. However, no correlation could be discerned between sarcomas evoked by 7,12-dimethylbenz(a)anthracene or benzo(a)pyrene and the inducible hydroxylase activity among the same inbred strains of mice. Genetic studies have indicated that inducibility in mice is under the control of a single genetic locus (11-14) and hybridization studies in hamster, mouse, and human cells indicate a closely coupled control mechanism for inducibility and basal AHH activity (15).

Kellermann, et al. (16) have observed that variation in extent of AHH induction in cultured human leukocytes is under genetic control and that the normal white population in the United States can be divided into three distinct phenotypes with low, intermediate and high degrees of inducibility. Two alleles and a single locus appear to be involved with the three groups representing homozygous low and high alleles and the intermediate heterozygote.

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The distribution followed the Hardy-Weinberg equilibrium, and gene frequencies of the low and high alleles in this population were 0.717 and 0.283 respectively.

Phenotype frequencies were 53%, 37%, and 10%. Family studies included all six possible crosses, and none of the offspring varied from expectations.

Huberman and Sachs (17) reached similar conclusions using a different test system.

Kellerman, et al. (18) have recently reported data from a study of fifty patients with bronchiogenic carcinoma which indicate that susceptibility to this disease is associated with higher levels of inducible aryl hydrocarbon hydroxylase activity.

have indicated that inducibility in mice is related to the content of aryl hydrocarbon hydroxylase activity in the liver.

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References

1. Miller, J.A.: Carcinogenesis by Chemical: An Overview - G.H.A. Clowes Memorial Lecture, Cancer Res. 30:559-576, 1970.
2. Gelboin, H.V., Kinoshita, N., Wiebel, F.J.: Microsomal Hydroxylases: Induction and Role in Polycyclic Hydrocarbon Carcinogenesis and Toxicity, Fed. Proc. 31:1298-1309, 1972.
3. Nebert, D.W., Goujon, F.M., Gielen, J.E.: Aryl Hydrocarbon Hydroxylase Induction by Polycyclic Hydrocarbons: Simple Autosomal Dominant Trait in the Mouse, Nature (New Biol.) 236:107-110, 1972.
4. Idem: Genetic Expression of Aryl Hydrocarbon Hydroxylase Induction. III. Changes in the Binding of N-Octylamine to Cytochrome P-450, Mol. Pharmacol. 8:651-666, 1972.
5. Levin, W., Conney, A.H., Alveres, A.P., et al.: Induction of Benzo(a)pyrene Hydroxylase in Human Skin, Science 176:419-420, 1972.
6. Juchau, M.R., Pederson, M.G., Symms, K.G.: Hydroxylation of 3,4-Benzpyrene in Human Fetal Tissue Homogenates, Biochem. Pharmacol. 21:2269-2272, 1972.
7. Busbee, D.L., Shaw, C.R., Cantrell, E.T.: Aryl Hydrocarbon Hydroxylase Induction in Human Leukocytes, Science 178:315-316, 1972.
8. Grover, P.L., Hower, A., Sims, P.: K-region Epoxides of Polycyclic Hydrocarbons: Formation and Further Metabolism of Benz(a)anthracene 5,6-Oxide by Human Lung Preparations, FEBS Lett 35:63-68, 1972.
9. Conney, A.H.: Pharmacological Implications of Microsomal Enzyme Induction, Pharmacol. Rev. 19:317-366, 1967.
10. Kouri, R.E., Rattie, H., Whitmire, C.E.: Evidence for a Genetic Relationship Between Susceptibility to 3-Methylcholanthrene-Induced Subcutaneous Tumors and Inducibility of Aryl Hydrocarbon Hydroxylase, J. Nat. Cancer Inst. 51:197-200, 1973.
11. Gielen, J.E., Goujon, F.M., Nebert, D.W.: Genetic Regulation of Aryl Hydrocarbon Hydroxylase Induction. II. Simple Mendelian Expression in Mouse Tissues In Vivo, J. Biol. Chem. 247:1125-1137, 1972.
12. Nebert, D.W., Gielen, J.E.: Genetic Regulation of Aryl Hydrocarbon Hydroxylase Induction in the Mouse, Fed. Proc. 31:1315-1325, 1972.
13. Thomas, P.E., Kouri, R.E., Hutton, J.J.: The Genetics of Aryl Hydrocarbon Hydroxylase Induction in Mice: A Single Gene Difference Between C57BL/6J and DBA/2J, Biochem. Genet. 6:157-168, 1972.
14. Thomas, P.E., Hutton, J.J.: Genetics of Aryl Hydrocarbon Hydroxylase Induction in Mice: Additive Inheritance in Crosses Between C3H/HeJ and DBA/2J, Biochem. Genet. 8:249-257, 1973.

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1. Miller, O.A.: Carcinogenesis of Chemicals. In: W. H. C. Chow, Ed., Carcinogenesis, Vol. 1, Plenum Press, New York, 1972.
15. Wiebel, F.J., Gelboin, H.V., Coon, H.G.: Regulation of Aryl Hydrocarbon Hydroxylase in Intraspecific Hybrids of Human, Mouse, and Hamster Cells, Proc. Nat. Acad. Sci. USA **60**:3580-3584, 1972.
16. Kellermann, G., Luyten-Kellermann, M., Shaw, C.R.: Genetic Variation of Aryl Hydrocarbon Hydroxylase in Human Lymphocytes, Amer. J. Hum. Genet. **25**:327-331, 1973.
17. Huberman, E., Sachs, L.: Metabolism of the Carcinogenic Hydrocarbon Benzo(a)pyrene in Human Fibroblast and Epithelial Cells, Int. J. Cancer **11**:412-418, 1973.
18. Kellermann, G., Shaw, C.R., Luyten-Kellermann, M.: Aryl Hydrocarbon Hydroxylase Inducibility and Bronchogenic Carcinoma, New Eng. J. Med. **289**:934-937, 1973.

19. Gelboin, H.V.: The Induction of Aryl Hydrocarbon Hydroxylase in Human Lymphocytes and its Relationship to Susceptibility to 3-Methylcholanthrene-Induced Subcutaneous Tumors and Induction of Aryl Hydrocarbon Hydroxylase in Human Lymphocytes.

20. Miller, O.A.: Carcinogenesis of Chemicals. In: W. H. C. Chow, Ed., Carcinogenesis, Vol. 1, Plenum Press, New York, 1972.

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#978 - MIMURA

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THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

February 13, 1974

Grant application No. 978

EPIDEMIOLOGY

To: The committee comprising Drs. Gardner, Sommers, Lynch

Subject: Goro Mimura, M.D., Institute of Constitutional Medicine,
Kumamoto University, Japan
New application No. 978
"The International Collaborative Twin Registry"

History

CTR sponsored planning meeting on International Collaborative Twin Studies at Miami, December 10 - 14, 1973. Seven of the participants (from New Zealand, United States, Belgium, Sweden, Australia, Japan and Finland) were invited to submit proposals. To date five have been received.

Request

The request is \$43,119 for the first year, plus two additional years at greater amounts.

Document Submitted

Enclosed is application, undated, received by CTR February 4, 1974.

Comment

Dr. Carl Seltzer has been asked to evaluate these proposals.

F.W.N.
F.W.N.

FWN:wg
Encl.

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THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

110 EAST 50TH STREET
NEW YORK, N. Y. 10022

(212) 421-8985

Application for Research Grant

Date:

(Use extra pages as needed)

1. Principal Investigator (give title and degrees):

Goro Mimura, M.D. Associate professor

2. Institution & address:

Institute of Constitutional Medicine, Kumamoto University.
4-24-1, Kuhonji, Kumamoto City, Japan.

3. Department(s) where research will be done or collaboration provided:

Department of Geriatrics.

4. Short title of study:

The International Collaborative Twin Registry.

5. Proposed starting date: 1st, July, 1974.

6. Estimated time to complete: 30th, June, 1977.

7. Brief description of specific research aims:

The purpose of the research is the registry of twins in Kumamoto Prefecture according to the method of international twin registry. And by using the twins who were registered, this study aims at evaluation of the interactions between genetic, environmental factors including smoking and their associations with disease, especially ischemic heart disease.

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Hereditary factors as well as environmental factors bring on ischemic heart disease. Therefore, to determine the influence of hereditary and environmental factors (including smoking) on health condition and disease, the study of monozygotic twins is most useful and even necessary.

9. Details of experimental design and procedures (append extra pages as necessary)

Experimental procedure of twin study

1) The registry procedure of twins

Three methods are considered for compiling a twin registry in Kumamoto Prefecture, Japan. One is based on census registration, the second on a mailing of questionnaires to all householders in Kumamoto Prefecture, and the third is based on solicitation of twin volunteers through local health centers. Kumamoto Prefecture has about 1,700,000 inhabitants in 450,000 households. Of these about 980,000 persons are 25 years of age or older. The rate of twin births in Japan is approximately 0.6 %, and among all twin births about 68 % are monozygotic. In the Kumamoto population consisting of people 25 years or older, about 2900 twin pairs would be expected. Of these about 1,900 pairs would be monozygotic. A unique feature of a twin register in Japan would be the possibility of finding twins reared apart from the time of birth. In the past it was not uncommon that twins were separated at birth by one of them being placed in another family. The best method to find such twins would probably be the use of birth certificates and census registration records in addition to the information from local health centers. Birth records can be linked to population records through residence data. In the case of using birth records, it is impossible to detect the present address of twins. Therefore, in order to detect twins, the best way is to use the census registration system in Japan.

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KUMAMOTO, JAPAN

But since World War II there are some disadvantages in this system, for detecting twin pairs. Namely, in the case where children get married the family register of children must be separated from the register of parents. If parents die, the family register of parents disappears from the present census registration. In the case where twin partners over the age of 25 got married and their family registers were separated from each other since 1948, the probability of the detection of twins over the age of 25 in the first stage of the examination will be very small.

Therefore, in the second stage of the examination I must go back to the past family register of their parents. As the pilot study I have investigated with the permission of the Ministry of Justice the past family registers of Kumamoto City which includes all family members. From the result of our pilot study, in order to find twin pairs from the investigation of family registers, at least two years and 4 person's work will be necessary. It will take about two minutes to check one family register. From our present study we found about one twin pair per one hundred past family registers for 460,000 people in Kumamoto City. Therefore, calculating from about 1,700,000 inhabitants of Kumamoto Prefecture, the number of past family registers will amount to 480,000. The rate of twin pairs which will be obtained from the past family registers will be one per cent, but the past family registers range from 1868 to 1947. About half of the twin pairs which we detected from the past family registers in Kumamoto City have already died. Therefore, the rate of finding twins from the investigation of the past family registers will be about 0.5 or 0.6 per cent. This percentage will not be very different compared with that obtained from the birth rate. After we have detected the twin pairs over 25 years old from the past family registers, as the second stage of the study we must investigate the new family registers of twins and we will find the address of twin pairs.

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KUMAMOTO, JAPAN

As there are about 480,000 past family registers in Kumamoto Prefecture, and as those are preserved in 10 cities, 30 towns and 58 village offices, the task of investigating all those family registers is not a small one. At the same time, we will gather information about twins from the health centers and other organizations.

After registry of twins, we will send the questionnaire to the twin partners.

2) The diagnosis of zygote in Japan

The degree of precisely diagnosing zygote is the fundamental problem in this study. The method of diagnosis which is used in my Institute is as follows. In order to diagnose the zygote, apart from anthropometrical measurement, the character of independent blood typing (ABO and MN blood type and Rh factor), fingerprinting, phenyl-thio-carbamide test (PTC test), mid digital hair test (with or without) and earwax test (dry earwax or wet earwax) will be necessary. In the PTC test, after administering PTC drops on the tongue, two groups are divided according to the presence or absence of bitterness. Final diagnosis of Zygote is made from the combination of all above-mentioned factors. The diagnosis of monozygotic twins is made in the case where the blood typing is concordant and other characters also are concordant. In all other cases, the diagnosis is made as dizygotic twin. There are certain criteria of concordance in fingerprinting. In other countries, it has been reported that the diagnosis of zygote based on the information record and the twins' opinions was the best method. But in Japan the condition is a little different in adult twins, because in the past people believed all monozygotic twins to be same-sexed. Therefore, in Japan in the case of adult twins, it is unknown what degree of probability of zygosity diagnosis I can get by only the twins' opinions of Zygosity. As for this problem, because the number of twins I have examined is not suffi-

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cient, at this stage I would like to refrain from a conclusion, but it is presumed that the degree of precisely diagnosing Zygote will decrease in Japan compared with that in other countries. For the above mentioned reasons, in the diagnosis of Zygote it is necessary to perform the examination of blood typing and other examinations if at all possible, in addition to twins' opinions. About one hour will be necessary to diagnose the Zygote. Therefore, two years will be necessary for this work.

3) The necessity of taking electrocardiogram

In Japan the death rate due to heart disease has increased during the past twenty years and heart disease is now third among the causes of death. It is presumed that this tendency is due to the increase of old age population, Europeanization of the mode of living and other circumstantial factors. In 1950 the death rate from ischaemic heart disease was 9.9 per cent per 100,000 population, but in 1962 and 1967 it was 19.8 and 30.3, respectively, but compared with the death rate from ischaemic heart disease in the U.S.A. the rate in Japan is only one tenth. And most of the ischaemic heart disease is without pain so typical angina pectoris and myocardial infarction are relatively few compared with those in Europe and U.S.A.. We may possibly miss a big number of ischaemic heart disease without pain when we use only this questionnaire in Japan. Therefore, I think electrocardiogram examination including exercise is necessary to cover all ischaemic heart disease cases. When the twin partner will come to our Institute, the electrocardiogram will be performed easily, but it will be difficult, for our research fellows to go to other cities, towns and villages.

As can be understood from the above mentioned reasons, the social condition of twins in Japan is different in several points compared with that in other countries. Therefore, in order to cooperate with the international collaborative study of twins it is necessary and it would be appreciated if the special character of twins in Japan could be given due consideration.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

The Institute of Constitutional Medicine is a five storey building (3240.0 m²). Half of the second floor belongs to our department alone. We have also the use of all the fifth floor which includes the laboratory. In the lab. there is the usual equipment e.g., autoanalyzer, spectrometer, fluorescentmeter, electromicroscope etc. As our department takes care of in and out patients in the adjoining University Hospital, we have the use of the lab. and equipment there.

11. Additional facilities required:

In order to examine and blood type the twins all over Kumamoto Prefecture we will require the use of the facilities of the different hospitals affiliated with our Institute. There are four such hospitals in Kumamoto Prefecture. We will also use and require the cooperation of 16 Health Centers in the Prefecture.

12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

1003545434

KUMAMOTO UNIVERSITY
INSTITUTE OF CONSTITUTIONAL MEDICINE

KUMAMOTO, JAPAN

The Institute of Constitutional Medicine is a live story

Curriculum Vitae

Date: Jan. 26, 1974

1. Name : Goro Mimura

2. Date of Birth: **REDACTED**

3. Sex : Male

4. Address **REDACTED**

5. Present Position and Institution: -

Associate Professor, Department of Geriatrics,
Institutional Medicine, Kumamoto University,
Japan.

6. Medical School Education:

In order to Kumamoto Medical College, 4 years.

7. Post Graduate Training after Internship:

Medical Staff, 3 years, Kumamoto University.

Assistant, 2 years, same Department.

Assistant Professor, 2 years, same Department.

Associate Professor, 12 years, same Department.

Goro Mimura

1003545435

KUMAMOTO UNIVERSITY

INSTITUTE OF CONSTITUTIONAL MEDICINE

KUMAMOTO, JAPAN

Curriculum Vitae

Date: Jan. 26, 1974

1. Name : Tomio Jinnouchi

2. Date of Birth: **REDACTED**

3. Sex : Male

4. Address : **REDACTED** **REDACTED**

5. Present Position and Institution:

Assistant Professor, Department of Geriatrics,
Institutional Medicine, Kumamoto University,
Japan.

6. Medical School Education:

Kumamoto Medical Collage, 4 years.

The postgraduate course, 4 years.

7. Post Graduate Training after Internship: Kumamoto University

Assistant, 3 months, Kumamoto University.

Assistant Professor, 8 years, same Department.

Tomio Jinnouchi

1003545436

KUMAMOTO UNIVERSITY

INSTITUTE OF CONSTITUTIONAL MEDICINE

KUMAMOTO, JAPAN

Curriculum Vitae

Date: Jan. 26, 1974

1. Name : Shoji Fukumitsu

2. Date of birth: REDACTED

3. Sex : Male

4. Address : REDACTED

REDACTED

5. Present Position and Institute:

Chief of Department of Cardiovascular
Disease and Main staff of Diabetes Clinic.

Yatsushiro Sogo General Hospital.

(Affiliated hospital to School of Medicine
and Research Institute of Constitutional
Medicine, Kumamoto University)

6. Medical School Education:

Graduated A from Medical School of
Kumamoto University after four years full
educational curriculum.

7. Postgraduate training after internship:

1955--1959 Medical Staff

Clinical training and research study in
cardiovascular disease and diabetes at
Department of Geriatrics, Research Institute
of Constitutional Medicine, Kumamoto University.

1959--1960 Assistant

Same as above

1960--1962 Clinival activities at Hiwatari
Clinic in Kagoshima city.Since December 25, 1962 at present position and
the Hospital.

1003545437

Shoji Fukumitsu

KUMAMOTO UNIVERSITY

INSTITUTE OF CONSTITUTIONAL MEDICINE

KUMAMOTO, JAPAN

Curriculum Vitae

Date : Jan. 26, 1974

1. Name : Haraguchi Yoshikuni

2. Date of Birth:

3. Sex : Male

4. Address :

5. Present Position and Institution:

Chief, Department of Cardiology, National
Kumamoto Hospital, Japan.

6. Medical School Education:

Kumamoto Medical Collage, 4 years.

The postgraduate course, 4 years.

7. Post Graduate Training after Internship

Assistant Professor, 1 years, Kumamoto University.

Chief, 2 years, Oita Prefectural Hospital.

Chief, 6 years, National Kumamoto Hospital.



1003545438

KUMAMOTO UNIVERSITY

INSTITUTE OF CONSTITUTIONAL MEDICINE

KUMAMOTO, JAPAN

Curriculum Vitae

Date: Jan, 26, 1974

1. Name : Keizo Kajiwara

2. Date of Birth:

REDACTED

3. Sex : Male

3. Sex : Male

4. Address :

REDACTED

REDACTED

5. Present Position and Institution:

Medical Staff, Department of Geriatrics,

Institutional Medicine, Kumamoto University,

Japan.

6. Medical School Education:

Kumamoto Medical Collage, 4 years.

7. Post Graduate Training after Internship:

Medical Staff, 2 years, Kumamoto University.

Chief, 2 years, Oita Prefectural Hospital.

Chief, 2 years, National Aomori Hospital.

Keizo Kajiwara

1003545439

KUMAMOTO UNIVERSITY

INSTITUTE OF CONSTITUTIONAL MEDICINE

KUMAMOTO, JAPAN

Publications:

- 1) Miyao, S. and Mimura, G.: On the morbidity in twins with special regard to malignant tumor and metabolic disease. Bull. of the Institute of Constitutional Medicine, Kumamoto Univ. 18:137 1967.
- 2) Mimura, G. Miyao, S., Koganemaru, K., Haraguchi, Y., Jinnouchi, T. and Hashiguchi, J.: Heredity of diabetes mellitus in Japan. Diabetes Mellitus in Japan. Excerpta Medica Intern. Series. No.221:83, 1970.
- 3) Mimura, G.: Epidemiology of diabetes in Asia, especially in Japan.: Diabetes, Excerpta Medica Intern. Series No.231:331, 1970.
- 4) Mimura, G. and Kodera, M.: Genetic study on essential hypertension. Japanese Circulation J. 37:61, 1973.
- 5) Mimura, G.: Study of twins with hypertension. Singapore Med.J. 14:278, 1973.

Kumamoto Medical College, Kumamoto, Japan

Medical Staff, 2 years, Kumamoto University

1003545440

14. First year budget:

A. Salaries (give names or state "to be recruited")	% time	Amount
Professional (give % time of investigator(s) even if no salary requested)		
Dr. Goro Mimura	30	-
Dr. Tomio Jinnouchi	25	-
Dr. Shouji Fukumitsu	15	-
Dr. Yoshikuni Haraguchi	20	-
Dr. Keizo Kajiwara	20	-
Technical		
Lab. Technician (1) (to be recruited)	100	3333
Typist (1) (to be recruited)	100	3333
Registration Staff (3) (to be recruited)	80	5000
Sub-Total for A		11666

B. Consumable supplies (by major categories)		
Paper and Printing Fee (Questionnaire etc)	3000	315172
Postage and Telephone	1500	66
Blood Typing Chemicals etc	1333	227
Sub-Total for B	5833	

C. Other expenses (itemize)		
Reward for cooperation (twins)	13330	
Travel Expenses	6665	
Sub-Total for C	19995	

D. Permanent equipment (itemize)		
Running Total of A + B + C	37494	

E. Indirect costs (15% of A+B+C)		
Sub-Total for D	0	
E	5624.8	
Total request	43118.8	dollars

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	11666	5833	53333	0	10624.8	81456.8
Year 3	6666	1660	53333	0	9248.85	70907.85

1003545441

16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
The study of twins	Institute of Constitutional Medicine, Kumamoto University	5000	Jan. 1. 1974 to Dec. 31. 1974

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Goro Mimura

Signature Goro Mimura Date 26th, Jan. 1974

Telephone 0963 63-1111 3762
Area Code Number Extension

Checks payable to

Goro Mimura, M.D., Associate professor

Responsible officer of institution

Typed Name Yoshitaka Harada

Mailing address for checks Department of Geriatrics,
Institute of Constitutional Medicine,
Kumamoto University, 4-24-1, Kuhonji,
Kumamoto City, Japan.

Title Director of Institute of

Signature Y. Harada Date 26th, Jan. 1974

Telephone 0963 63-1111 3711
Area Code Number Extension

1003545442

#953 - RANTASALO

1003545443

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

January 16, 1974

Grant application #953

EPIDEMIOLOGY

To: The committee comprising Drs. Gardner, Jacobson and Lynch

Subject: Ilari Rantasalo, M.D., University of Helsinki, Finland
New application No. 953
"The Finnish Twin Registry"

History

Seven of the participants in a planning meeting on International Collaborative Twin studies sponsored by CTR in Miami Beach on December 10 - 14, 1973 were invited to submit applications for grants. The enclosed is the first to arrive.

Request

For the first year, application #953 requests 244,940 Fmk, approximately equal at present to \$61,235.

Roughly the same amount is budgeted for second and third years. The entire study is estimated to require "over 10 years".

Documents Submitted

Enclosed is application dated 9.1.1974 (received by us January 14, 1974) with Appendices I, II and III.

FWN:gh

Encls.


F.W.N.

1003545444

#953

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

Date: 9.1.1974

1. Principal Investigator (give title and degrees):

Dr. Ilari Rantasalo M.D., Professor and Chairman
Department of Public Health Science
University of Helsinki

2. Institution & address:

Department of Public Health Science
University of Helsinki
Haartmaninkatu 3
SF 00290 Helsinki 29, FINLAND

3. Department(s) where research will be done or collaboration provided:

see the point two

4. Short title of study:

The Finnish Twin Registry

5. Proposed starting date: 01.01.1974

6. Estimated time to complete: over ten years

7. Brief description of specific research aims:

It is our aim to research the connection of some diseases (coronary heart disease, chronic lung diseases, malignant tumors etc) and various environmental factors (tobacco smoking, alcohol drinking, stress etc) using twin metod:

- a) as a part of an international study
- b) creating at the same time a basic register for further studies.

Program of the year 1974:

- a) the creation of the address registry of the same sex twins in Finland.
- b) the working out of the mailed "Miami" questionnaire including the optional questions.

JAN 14 1974

1003545445

8. Brief statement of working hypothesis:

2.

Using the twin method it is possible to study environmental factors so that the genetic factors are standardized.

Working hypothesis was documented in San Juan Report:

"Twin Registries in the Study of Chronic Disease"

(Acta Medica Scandinavica, supplementum 523, 1969)

9. Details of experimental design and procedures (append extra pages as necessary)

Compilation of the address registry

The central registry of population records all Finnish people, who live in Finland. This information is recorded on magnetic tapes in two registries: the personal registry and the real estate registry.

The personal registry contains among other things the following particulars:

- personal number (birthdate plus a unique individual number)
- sex
- native place of birth
- surname and first name
- maiden name for married women
- symbol for native place
- civilian estate
- from the beginning of 1975 also the personal numbers of parents and children will be on the tape.

The real estate registry contains among other things the address.

The procedure of collecting the twin registry will be the following. All people with the same birthdate, the same sex, the same surname (in the case of women the maiden name will be used), and the same native place of birth will be grouped together. Starting from this tape it is then a purely computerized procedure to get hold of the addresses by matching the personal registry with the real estate registry. This list of people (= candidates) certainly contains false pairs, i.e. pairs where names, place of birth and birthdate coincide by chance, which will probably happen frequently only in larger localities. By the help of a mailed questionnaire it is possible to pick up the twin pairs from the mass of the candidates and to get basic data at the same time. From the beginning of 1975 it is possible to repair this twin tape using the personal numbers of mother: twins are people who have the same birthdate (or adjoining day) and the same mother.

The size of the sample (all people available in Finland)

It is profitable to create the address registry from all age groups at the same time, though we intend at the beginning to study only people over 20 years of age. The size of the twin population appears from the following table.

see appendix number one

1003545446

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

The Department of Public Health Science has extra space sufficiently for the Finnish Twin Registry.

The department has a terminal of the computer of the university (UNIVAC 2000). The punching and the analysis of the material are possible to perform with the help of the department's facilities.

11. Additional facilities required:

12. Biographical sketches of investigator(s) and other professional personnel (append): appendix II

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available):

appendix III

1003545447

14. First year budget:

A. Salaries (give names or state "to be recruited")
Professional (give % time of investigator(s)
even if no salary requested)

Principal investigator

% time

Amount

5 %

Designer, 3000 Fmk/kk x 12

60 %

36 000 Fmk

Technical

two clerks 1500 Fmk/kk x 10 x 2

100 %

30 000

three clerks 1300 Fmk/kk x 9 x 3

100 %

35 100

(one for punching and two for
coding)

- social security 8 %

8 090

Sub-Total for A

109 190

B. Consumable supplies (by major categories)

Postage 40 000 x 2 x 0,6 Fmk

48 000

Envelopes 40 000 x 4 x 0,03 Fmk

4 800

Printing of forms 40 000 x 2 x 0,3 Fmk

24 000

Sub-Total for B

76 800

C. Other expenses (itemize)

Computer costs, candidates of twins + addresses

27 000

Sub-Total for C

27 000

Running Total of A + B + C

212 990

D. Permanent equipment (itemize)

Sub-Total for D

31 950

E

E. Indirect costs (15% of A+B+C)

Total request

244 940 Fmk = 61 235 \$

15. Estimated future requirements:

(1 \$ = 4,00 Fmk 8.1.1974)

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	128 300	57 600	38 000	—	33 600	257 500 Fmk
Year 3	128 300	57 600	18 000	—	30 600	234 500 Fmk

1003545448

16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
The Finnish Twin Registry	own institution	2000 \$	1974
Married couple study	Yrjö Jahnsson's foundation	1750 \$	1974

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
The Finnish Twin Registry	Academy	25 000 \$?	1975-1976

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Ilari Rantasalo
 Signature *Ilari Rantasalo* Date 9.1.1974
 Telephone Helsinki 418 511
Area Code Number Extension

Checks payable to Department of Public Health Science
University of Helsinki

Responsible officer of institution

Typed Name Ilari Rantasalo
 Title Professor and Chairman
 Signature *Ilari Rantasalo* Date 9.1.1974
 Telephone Helsinki 418 511
Area Code Number Extension

Mailing address for checks

Haartmaninkatu 3

SF- 00290 Helsinki 29

1003545449

Appendix I (The Finnish Twin Registry)

The table is an estimation based on number of in Finland living people of the same ten-years groups, number of twin births in the ten-years groups, mortality of twins according to partner.

It is also estimated that 63 % of the twin pairs are of the same sex and that 33 % of the same sex are identical.

ESTIMATED NUMBERS OF TWIN PAIRS OF THE SAME SEX IN FINLAND

YEAR OF BIRTH	MONOZYGOTIC		DIZYGOTIC	
	MALE	FEMALE	MALE	FEMALE
1891-1900	58	117	115	235
1901-1910	184	271	368	542
1911-1920	351	452	703	905
1921-1930	401	481	802	962
1931-1940	738	732	1476	1464
1941-1950 x)	617	594	1233	1188
total	2349	2647	4697	5296
	4996		9993	

TOTAL NUMBER OF PAIRS 14 989

x) migration of people from Finland (mainly to Sweden) has reduced this age group.

Follow-up studies

Morbidity

It is possible to get information (except using questionnaires) from the Central Medical Board (CMB), from the Institute of Health Insurance (IHI), from the Finnish Cancer Registry, from the Kidney Disease Registry, from the Malformation Registry, and from the Coronary Registries of some larger towns.

The CMB records all people, who have left hospital (surviving or dead).

The IHI records all people , who have got money or service as compensation for a disease.

Mortality

The Central Statistical Office records all death certificates.

Some other things

The Administration of the State has many registries on magnetic tapes. The most interesting of them are registries consisting among other things crimes, traffic offenses and earned income.

The follow-up with all these means is possible purely with the help of computers.

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Biographical sketches:

Principal investigator (Ilari Rantasalo)

- born 1916
- licenciate in medicine 1946
- doctor of medicine (= dissertation) 1948
- specialist in:
 - pediatrics
 - infectious diseases
 - laboratory investigation (microbiology)
 - public health
- assosiate professor in pediatrics 1959
- principal occupations:
 - 1948-1963: Head of the bacteriological department of the State Serum Institute
 - 1964- : Professor and Chairman of the Department of Public Health Science
(before 1971 the name was called Department of Hygiene)

Designer (Markku Koskenvuo)

- born 1945
- licenciate in medicine 1972
- some substitutes as assistant physician in departments of internal medicine 1968-1972
- assistant of Department of Public Health Science 1972-.

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Appendix III (The Finnish Twin Registry)

Publications:

Rantasalo:

The published papers (list of them) during Rantasalo's chairman period in the department of Public Health Science are enclosed (from years 1966-1972).

Koskenvuo:

A married couple study, a method for measuring environmental factors adapting to coronary heart disease. (Dissertation, unpublished)

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